RIBONUCLEASE ACTIVITY OF α- AND β-MMC, TWO RIBOSOME-INACTIVATING PROTEINS ISOLATED FROM THE SEEDS OF *MOMORDICA CHARANTIA*

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A THESIS SUBMITTED IN PARTIAL FULFILMENT
OF THE REQUIREMENT FOR THE DEGREE OF
MASTER OF PHILOSOPHY
IN
BIOCHEMISTRY

JULY 1996

DEPARTMENT OF BIOCHEMISTRY
THE CHINESE UNIVERSITY OF HONG KONG

ACKNOWLEDGEMENTS

First of all, I would like to express my most sincere gratitude to my supervisor, Dr. Wing-Ping Fong, for his continuous guidance throughout my study and valuable advice in the preparation of this thesis.

Many grateful thanks are due to Dr. T. B. Ng and Dr. S. K. Kong, and especially my external examiner, Dr. W. N. Leung, Department of Biology, Hong Kong Baptist University, for their willingness to be members of my thesis committee.

Last, but not the least, I would also like to thank all the staff and students in the Department of Biochemistry for their help during my postgraduate study.

Abstract

α- and β-Momorcharins (α- and β-MMCs) are type I ribosome-inactivating proteins isolated from the seeds of *Momordica charantia*. Both of them are glycoproteins of molecular weight about 30kDa and with high pI values (pI~9). Besides the well known abortifacient effect, they have also been demonstrated to have anti-tumor, immunosuppressive and anti-HIV effects.

In the present study, the MMCs were purified by a modified purification method which consisted of two ion-exchange chromatographies, namely, DEAE-cellulose and Mono-S FPLC chromatography. By using this purification procedure, the purification time was reduced to two days while the yield of both MMCs remained satisfactory. The purified MMCs were known to have N-glycosidase activity and this activity required the ribosomal protein for its action. MMCs were also shown to possess ribonuclease activity by using tRNA as substrate. Further investigation of the substrate specificity of the reaction was achieved by using the various polyhomoribonucleotides. The result showed that MMCs acted specifically towards polyU, but had little or no effect on polyA, polyC and polyG. Analysis of the reaction product by DEAE-Sepharose and Mono-Q FPLC chromatography suggested that the reaction was ribonucleolytic, but not N-glycosidic, in nature.

During the purification of MMCs, another protein that possess ribonuclease activity was also isolated, named as ribonuclease-MC (RNase-MC). RNase-MC was eluted by a much higher sodium chloride concentration in the Mono-S FPLC chromatography, when compared with the MMCs. The RNase-MC also acted on tRNA, but the substrate specificity was quite different. Besides acting on polyU, RNase-MC also had significant activity on polyC. The pH optimum for the activity was pH 6.0 which was slightly higher than that of both MMCs (pH 5.5). These suggested that the ribonuclease activity detected in MMCs was not due to the presence of RNase-MC in the preparation.

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Chapter 1 INTRODUCTION

1. Ribosome-Inactivating Proteins (RIPs)

1.1 Enzymatic Activity of RIPs

Ribosome-inactivating protein (RIP) is a group of proteins that inhibits protein synthesis in eukaryotic cells (Stirpe *et al.*, 1992). They selectively and irreversibly inactivate the 60S eukaroytic ribosomal subunits and terminate the peptide elongation process. At present, two different enzymatic mechanisms have been identified, namely, the N-glycosidase activity and the ribonuclease (RNase) activity (Fong *et al.*, 1991). Most plant and bacterial RIPs, for example, luffin, ricin and *Viscum album* agglutinins (VAA), act as N-glycosidase whereas the fungal RIPs like α–sarcin act as ribonuclase. Either cleavage results in the damage of ribosome and makes them unable to bind elongation factor 2 (EF-2). The ultimate consequence is termination of protein synthesis.

1.1.1 N-glycosidase activity

The N-glycosidase activity has been extensively investigated in the last decade. Endo and his colleague, using ricin as an example, were the first to discover the N-glycosidase activity of plant RIPs. They found that RIPs act on the rRNA to release a single adenine molecule (Endo *et al.*, 1987). The N-glycosidase activity is highly specific. Only a single N-glycosidic bond between adenine and ribose at a specific nucleotide A-

4324 of the 28S rRNA is hydrolytically cleaved. This modification of the rRNA makes the nearby phosphodiester backbone susceptible to the attack of aniline under acidic condition. Therefore, after subsequent acid aniline treatment, the RIP-modified 28S rRNA released a diagnostic RNA fragment of size about 400 base pair which can be easily observed upon electrophoretic analysis. This small diagnostic RNA fragment is known as the Endo's band or the Endo's fragment. Such specific depurination reaction in the 28S rRNA leads to conformational changes of the ribosome in the vicinity of ribosomal proteins L5 and L14 (Paleologue *et al.*, 1986; Terao *et al.*, 1988) which are located at the boundary between the 60S and 40S ribosomal subunits (Uchiumi *et al.*, 1985), a region which interacts with mRNA (Takahashi *et al.*, 1981). This RIP-induced conformational change could account for its inhibition on the protein-synthesizing activity of ribosomes.

In addition to the highly specific N-glycosidase cleavage at position A-4324, recently some non-specific depurination has also been reported for some RIPs (Barbieri *et al.*, 1992). It has been reported that saporin from different origins can release more than one mole of adenine from one mole of ribosome (Table 1.1). For example, saporin-L1 and saporin-R2, originated from leave and root respectively, can release as much as 30 moles of adenine per mole of ribosome. On the other hand, pokeweed antiviral protein (PAP) and trichokirin can also release more than two moles of adenine per mole of ribosome. Although multiple depurination occurs, however, the N- glycosidase activity of

Table 1.1 Depurination of rat liver ribosome by RIPs (Barbieri *et al.*, 1992).

RIPs	Adenine released (pmol/pmol of ribosomes)
Saporin-L1	36.3
Saporin-L2	26.1
Saporin-R1	2.8
Saporin-R2	32.0
Saporin-R3	11.9
Saporin-S5	1.5
Saporin-S6	2.5
Saporin-S8	1.3
Saporin-S9	5.6
PAP-R	2.7
Trichokirin	2.0

the various RIPs seems to be stronger at the rRNA sequence around the A4324 position rather than at other positions (Barbieri *et al.*, 1992).

The multi-depurinating properties of saporins are not limited to rRNA substrate, instead, it also occurs in some other ribo- and deoxyribopolynucleotides (Barbieri *et al.*, 1994) (Table 1.2). The effects of the saporins on mRNA and tRNA suggests that they may inhibit protein synthesis not only by altering the ribosome, but also through its effects on mRNA and tRNA (Barbieri *et al.*, 1994). It has also been reported that saporins can directly inhibit the replication of viruses by damaging their genomic or messenger RNA (Barbieri *et al.*, 1994). Such enzymatic activity might be the basis of the antiviral activity of the RIPs.

Table 1.2 Effect of saporin L-1 on various adenine containing substrate (Barbieri *et al.*,1994).

Substrate	**Adenine released (pmol)**
PolyA	2,038
Globin mRNA (rabbit reticulocytes)	1,587
DNA (herring sperm)	747
***Bryonia dioica* polyA-RNA**	524
***Escherichia coil* rRNA (16S + 23S)**	435
***Saccharomyces cerevisiae* $tRNA^{phe}$**	425
Tobacco mosaic virus (TMV) genomic RNA	371
Bacteriophage MS 2 genomic RNA	336

1.1.2 Ribonuclease Activity

While most, if not all, plant and bacterial RIPs inactivate ribosome through their N-glycosidase activity, fungal RIPs, for example, α-sarcin derived from the mold *Aspergillus giganteus,* inactivate ribosome by their RNase activity. The action of α-sarcin towards intact ribosome is also highly specific: only a single phosphodiester bond in the 28S rRNA is hydrolyzed, releasing a small α-fragment. The cleavage site of α-sarcin is located within a highly conserved, purine rich, single strand loop of rRNA. The phosphodiester bond cleaved is just adjacent to the N-glycosidic bond cleaved by the plant and bacterial RIPs (Fernandez-Puentes & Vazquez, 1977; Hobden & Cundliffe, 1978) (Figure 1.1). Such activity does not affect 5S, 5.8S and 18S rRNA. The RNase activity occurs in both cell-free system as well as intact cell. For example, α-sarcin has been reported to cleave the 28S rRNA in *Xenopus* oocytes after microinjection (Ackerman *et al.*, 1988).

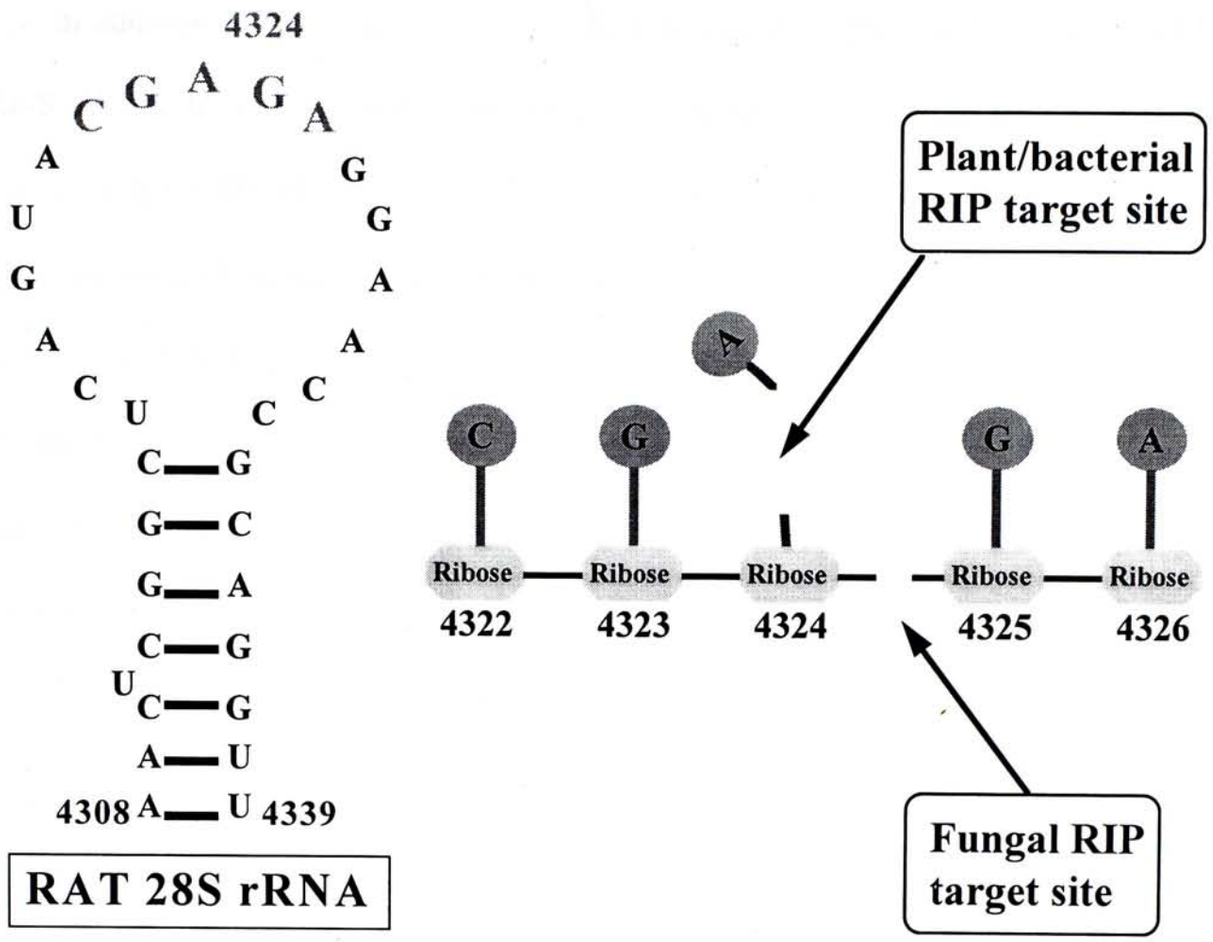

Fig. 1.1 The cleavage site of N-glycosidase and RNase activities of RIPs.

Besides α-sarcin, other fungal RIPs, such as restrictocin and mitogillin, two small basic proteins originated from the mold *Aspergillus restrictus*, also inhibit protein synthesis through their RNase activity: exactly the same phosphodiester bond in the 28S rRNA are cleaved (Fando *et al.*, 1985). Structurally, these two RIPs are also similar to α-sarcin. Their primary sequences are about 86% homologous with that of α-sarcin (Lopez-otin *et al.*, 1984). Their secondary and tertiary structures also resemble those of α-sarcin.

In addition to the fungal RIPs that show RNase activity, recently, a plant RIP, luffin-S isolated from the seed of *Luffa cylindrica*, has also been demonstrated to possess RNase activity on 28S rRNA (Gao *et al.*, 1993). Luffin-S has a molecular weight around 10kDa. Amino acid sequence analysis shows that it has little homology with α-sarcin or RNase A. Luffin-S displays an inhibitory effect on cell-free protein synthesis comparable with that of trichosanthin and α-sarcin. Enzymatically, luffin-S acts on the 28S rRNA and cleaves one single phosphodiester bond, with the release of a small RNA fragment, known as the S-fragment, similar in size to the α-fragment found in the action of α-sarcin. However, the exact cleavage site of luffin-S has not been determined.

1.2 Types of RIPs

Traditionally, RIPs are classified into two classes according to the number of subunits they possessed (Stirpe *et al.*, 1992). Type I RIPs possess one single polypeptide chain while type II RIPs consist of two polypeptide chains. Most of the RIPs examined thus far belong to type I RIPs. They have a single enzymatic subunit (active A-chain) with molecular weight between 26-30kDa. Type I RIPs have low toxicity towards intact cell but they are highly toxic in cell-free protein synthesis system. Examples of type I RIP include gelonin, saporin and trichokirin (Table 1.3).

Type II RIPs possess two polypeptide chains. One chain is the enzymatic subunit (active A-chain) and the other is the galactose specific lectin subunit (binding B-chain). Both of these subunits have molecular weight around 26-32kDa. These two chains are linked together by a disulfide bridge as well as some hydrophobic interaction. These proteins can enter the cell through the interaction of the binding B-chain with the cell membrane. Consequently, they are potent toxins and have high toxicity in both intact cell and cell-free systems. Some examples of type II RIPs are ricin, abrin and viscumin (Table 1.4).

Table 1.3 Examples of type I ribosome-inactivating proteins.

Plant name	Source	RIPs discovered
Caryophyllaceae		
Dianthus caryophyllus	leaves	Dianthin 30
(carnation)		Dianthin 32
Lychnis chalcedonica	seeds	Lychnin
Saponaria officinalis	seeds	Saporin 5
(Soapwort)		Saporin 6
Phytolaccaceae		
Phytolacca americana	leaves	Pokeweed antiviral protein (PAP)
(pokeweed)	summer leaves	PAP II
	seeds	PAP-S
	roots	PAP-R
	cels	PAP-C
Euphorbiaceae		
Gelonium multiflorum	seeds	Gelonin
Manihot palmata	seeds	Mapalmin
Cucurbitaceae		
Bryonia dioica	leaves	Bryodin-L
(white bryonyl)	roots	Bryodin
Citrullus colocynthis	seeds	Colocin 1
		Colocin 2
Luffa cylindrica	seeds	Luffin a
(sponge gourd)		Luffin b
Momordica charantia	seeds	Momordin
(bitter gourd)		MAP-30
Trichosanthes kirilowii	roots	Trichosanthin
		TAP-29
	seeds	Trichokirin

Table 1.4 Examples of type II ribosome-inactivating proteins.

Plant name	Source	RIPs discovered
Euphorbiaceae		
Ricinus communis **(castor bean plant)**	seeds	Ricin
Fabaceae		
Abrus precatorius **(jequirity)**	seeds	Abrin
Viscaceae		
Viscum album **(mistletoe)**	leaves	Viscumin
Passifloraceae		
Adenia digitata **(kilyambiti plant)**	roots	Modeccin
Adenia volkensii	roots	Volkensin

Recently, some RIPs are found to contain four subunits and thus represent an additional type of RIP. Consequently, an extended classification of RIPs is proposed recently (Citores *et al.*, 1993). Altogether three different types of RIPs can now be differentiated, namely, type I, type II and type IV, according to the number of subunits present. Moreover, type II and type IV can be further subdivided into two subclasses, toxic and non-toxic. Unlike the type I RIPs, type II RIPs possess the binding B subunit and hence have always been regarded as potent toxins, not only in *in vitro* system, but also in *in vivo* intact cell condition. However, recently, two novel, relatively non-toxic

type II RIPs, ebulin 1 (Girbes *et al.*, 1993) and nigrin b (Girbes *et al.*, 1993) were discovered. They show relatively low toxicity on intact cell, but are capable of arresting protein synthesis in cell-free system at nanomolar concentration. Ebulin 1, a type II RIP isolated from the leaves of *Sambucus ebulus L.* (*Caprifoliaceae*) is non-toxic to mice up to 2 mg/kg of body weight. It is also non-toxic to cultured cells which are highly sensitive to other type II RIPs such as ricin and abrin. Structurally, ebulin 1 is similar to the typical type II RIPs. Ebulin 1 consists of one catalytic A-chain and one D-galactose binding lectin B-chain. The size of both chains is roughly the same as that of other type II RIPs. Moreover, the N-terminal amino acid sequences of both the A- and B-chains of ebulin 1 display a high sequence homology with other type II RIPs. Like other RIPs, ebulin 1 also shows N-glycosidase activity against eukaroytic ribosome, thus, it releases the diagnostic Endo's RNA fragment after subsequent acid aniline treatment.

The low toxicity of ebulin 1 towards intact cell and whole animals may be a result of the failure in intracellular routing (Van Deurs *et al.*, 1986), such as premature degradation, inability to translocate through the endosomal membrane or difficulty in reducing the disulfide bridge that links the A- and B-chains together. Alternatively, the lack of toxicity could also be a consequence of the association of the dimer that result in the formation of inactive oligomer which fail to show the powerful enzymatic activity of the A-chain. Whatever the reason, ebulin 1 is relatively non-toxic and thus represents a new subclass of type II RIPs.

Type IV RIP is another new class of RIP in the extended classification system. Type IV RIPs are four chains agglutinins that was first discovered 20 years ago (Saltvedt *et al.,* 1976). They have been reported to inhibit *in vitro* protein synthesis (Citores *et al.,* 1993). Structurally, these agglutinins are composed of four polypeptide chains, including two enzymatic A-chains with molecular weight around 32kDa and two binding B-chains with molecular weight around 34kDa. Some examples of type IV RIPs are *Ricinus communis* agglutinins (RCA), *Abrus precatoruis* agglutinins (APA) and *Viscum album* agglutinins (VAA).

Early reports on plant protein inhibitors of protein synthesis suggested that the four-chains agglutinins RCA, APA and VAA inhibit *in vitro* translation in mammalian systems (Citores *et al.*, 1993). The IC_{50} values, that is, concentration giving 50% inhibition, for inhibition of translation in cell-free system are 5.5, 6 and 12 ng/ml for RCA, APA and VAA respectively. These values are close to those described for the traditional types I and II RIPs. The A-chain of these agglutinins are N-glycosidases acting on eukaryotic rRNA. The strong N-glycosidase activity enables the release of the diagnostic Endo's RNA fragment after acid aniline treatment. Among the three agglutinins, only VAA displays a mild toxicity towards mice, the toxicity is approximately ten-fold less than that of the common type II RIP, ricin. However, neither RCA nor APA are toxic to mice. Consequently, four-chains agglutinins can also be divided into two subclasses: toxic and non-toxic type IV RIPs. To sum up, the newly

proposed classification scheme of RIPs identify three types of RIPs, and for both type II and type IV RIPs, it can further be divided into toxic and non-toxic ones (Citores *et al.*, 1993) (Table 1.5).

Table 1.5 Extended classification of plant RIPs.

Type	Sub-classes	Examples
Type I (one chain)	non-toxic	Gelonin, saporin, trichosanthin and luffin a
Type II (two chains)	non-toxic	Ebulin 1, nigrin b
	toxic	Ricin, abrin, modeccin and viscumin
Type IV (four chains)	non-toxic	RCA, APA and *M. charantia* agglutinin
	toxic	VAA

1.3 Interaction of RIPs with Cell

RIPs can act on rRNA and DNA through different enzymatic activities, however, exactly how these toxins get into contact with their target substrate is still a question to be solved. The entry mechanism of RIPs into their intracellular sites has been the subject of many studies. The interest in the internalization of these toxins is growing because an understanding of the mechanism is essential for the possible therapeutic utilization of the toxins. So far, most of the studies has been focused on type II RIPs. It appears that more than one internalization mechanism are involved.

1.3.1 Internalization of Type II RIPs

The first step in the internalization process is the binding to the cell surface. RIPs bind to certain receptor sites on the cell membrane, the number of binding sites for the RIP varies from one cell type to another. However, the total binding capacity for a given RIP does not correlate with the sensitivity of the cell to that particular RIP. Different surface receptors with dissimilar affinities are present on the cell surface. These different receptors are responsible for the intracellular routing of the RIP (Barbieri *et al.*, 1993).

There are two RIP recognition processes. The first one is through the galactose specific binding B-chain of type II RIPs. Every cell-type can recognize the B-chain

because all cell types possess galactose-containing glycoproteins or glycolipids on their membrane. The second recognition process involves the interaction of cell receptors with the carbohydrate side chains in the RIP. For example, both the A- and B-chains of ricin possess mannose-containing oligosaccharide groups (Skilleter *et al.,* 1985; Magnusson *et al.,* 1993) and these carbohydrate side chains may also play a role in the binding of the RIP to the cell.

Following the process of recognition and binding of the RIP to the cell surface, internalization takes place by endocytosis. Type II RIPs do not normally cross the plasma membrane directly, instead, they enter the cytosol by endocytosis, a temperature-dependent and energy-consuming process. Endocytosis of RIPs is a receptor-mediated process and may occur by way of clathrin coated pits which are specialized depressions on the cell surface. Clathrin is a fibrous protein that provides the mechanical strength and flexibility required when a vesicle is pinched off from the membrane (Crowther *et al.,* 1981). Mannose-containing RIPs can utilize this way of endocytosis after being bound to mannose receptors localized on the clathrin coated pits (Magnusson *et al.,* 1991). However, RIPs can also utilize the uncoated pits for endocytosis. After endocytosis, both the coated and uncoated pits become smooth-surfaced vesicles called endosome.

RIPs in the endosome is ready for transport to intracellular compartment. The transport and sorting of macromolecules inside the cell consist of the endocytic and the

biosynthetic/secretary pathways, representing the afferent and efferent arm respectively (Barbieri *et al.,* 1993). The endocytic pathway comprises early and late endosomes and lysosomes whereas the biosynthetic/secretary pathway includes the endoplasmic reticulum and the Golgi complex. Some internalized RIPs are directed to the lysosomes and degraded whereas others are transported from endosomes to the Golgi complex by acidic vesicles before entering the cytosol (Sandvig *et al.,* 1984). RIPs with intracellular sites of action may reach their cytosolic target from a number of vesicular and tubular compartments. However, it is still unclear where these RIPs enter the cytosol, although it has been demonstrated that the site of transfer across membrane may be different for different RIPs.

1.3.2 Internalization of Type I RIPs

The entry mechanism of type I RIP is not well known. Nevertheless, some hypotheses have been formulated (Barbieri *et al.*, 1993). Glycosyl residues are present in most type I RIPs. These residues may bind to the carbohydrate receptors, for example, the mannose receptor, on the cell membrane and thus enable the RIP to be internalized. However, the low cytotoxicity of type I RIPs suggested that receptor-mediated internalization is not very efficient. In fact, saporin-S6 with no sugar moiety is relatively more toxic to many cell types than the glycosylated type I RIPs (Battelli, *et al.*, 1992). Therefore, it appears that there is no correlation between the toxicity to cell and the

presence of sugar residues in a given RIP (Battelli *et al.*, 1992). This also suggests the presence of a receptor-independent mechanism of internalization in the non-glycosylated type I RIPs. This type of endocytosis can take place in both coated and uncoated pits which result in the non-selective uptake of RIPs. It is less efficient than endocytosis which utilize specific surface binding sites. Saporin and possibly other type I RIPs that are non-glycosylated could be internalized by this kind of endocytosis into the cells (Madan *et al.*, 1992).

To conclude, RIPs can be taken up by cells through two mechanisms. The first type of endocytosis depends on the binding of RIPs to either the galactosyl residues (type II RIPs) or the mannose receptors on the cell membrane (both type I and type II RIPs). The other is receptor-independent endocytosis which probably is responsible for the internalization of non-glycosylated type I RIPs.

2. General Properties of α- and β- Momorcharins (MMCs)

α- and β- Momorcharins (α- and β-MMCs) are two type I RIPs isolated from the seeds of *Momordica charantia* (Yeung *et al.,* 1985), a well known Chinese medicinal herb. Taxonomically, *Momordica charantia* belongs to the family of *Cucurbitaceae.* Different parts of this plant, including seeds, fruits, vines, leaves and root, have been used in Chinese medicine since ancient times for the treatment of heat-stroke, dysentery, thirst from febrile symptoms as well as skin ulcers (Jiangsu *et al.,* 1979). In addition, this plant is also well known to have abortifacient effect (Morton *et al.,* 1967). However, the exact active components responsible for these effects and the action mechanisms behind were unknown at that time.

α and β-Momorcharins was purified from the seeds of *Momordica charantia* about ten years ago (Yeung *et al.,* 1985) and is believed to be responsible for the pharmacological effects of *Momordica charantia.* These two proteins are active abortifacient proteins and induce mid-term abortion in mice (Yeung *et al.,* 1985). This abortifacient activity has also been reported in several proteins isolated from other members of the same family of plant, for example, *Trichosanthes kirilowii* (Jin *et al.,* 1981), *Trichosanthes cucumeroides* (Yeung and Li, 1986) and *Momordica cochinchinesis* (Yeung *et al.,* 1980). Among them, trichosanthin (TCS) isolated from the root tubers of *Trichosanthes kirilowii* has been investigated most extensively. It has been reported to

have a wide spectrum of biological activities, such as anti-tumor cell growth (Tsao *et al.*, 1986), suppression of immune responses (Poon, 1981; Leung *et al.*, 1986) and inhibition on human immunodefficiency virus (HIV) replication (McGrath *et al.*, 1989).

2.1 Physical and Chemical Properties of MMCs

α- and β-MMCs possess similar physical and chemical properties. Both of them are basic glycoproteins and they are also similar with respect to molecular weight, isoelectric point (pI), amino acid composition as well as secondary structure. α- and β-MMCs possess 1.6% and 1.3% of neutral sugar respectively, according to the analysis by the phenol-sulfuric acid method (Yeung *et al.*, 1987). In this regard, the MMCs are different from TCS as the latter has been shown to be devoid of any carbohydrate moiety.

Judging from sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE), the molecular weights of α- and β-MMCs were determined to be about 30kDa (Yeung *et al.*, 1986), slightly higher than that of TCS which is about 26kDa. A more accurate calculation from the amino acid composition showed that the molecular weights of α- and β-MMCs are 29,092 (Ho *et al.*, 1991) and 28,000 (Yeung *et al.*, 1987) respectively.

Both MMCs are basic proteins. β-MMC is slightly more basic than α-MMCs. Their pI values are around 8.5-9.0. In this regard, they are also very similar to TCS which also has a high pI value of 9.4 (Kubota *et al.,* 1986).

The amino acid composition of α- and β-MMCs have been determined (Table 1.6) (Yeung *et al.,* 1986; Kubota *et al.,* 1986). They consists of 244 and 231 amino acids respectively. The composition of these two MMCs are quite similar to that of TCS. None of them possess any cysteine residues, therefore, no inter- or intra-molecular disulfide bridge can be formed. Also, these three proteins contain large amount of Asp/Asn and Glu/Gln amino acid residues and small amount of basic residues such as Lys and Arg. Based on circular dichroism analysis, their secondary structures are also very similar (Kubota *et al.,* 1986). They contain a moderate amount of α-helix (about 30%) and β-sheet (40-60%). However, they do not have any β-turns. Their structures are stable within a wide pH range between 5 and 9. Although the physical and chemical properties of α-, β-MMCs and TCS are very similar to each other, they are immunochemically distinct. It seems that there are distinct sites regulating the immunological and biological activities in these proteins (Ng *et al.,* 1993).

Table 1.6 Amino acid composition of TCS, α- and β-MMC (Kubota *et al.*, 1986).

Amino acid	TCS	α-MMC	β-MMC
Asx	29	28	27
Thr	14	15	19
Ser	23	16	16
Glx	19	23	21
Pro	8	11	9
Gly	10	15	8
Ala	24	24	22
Cys	0	0	0
Val	11	15	15
Met	4	3	0
Ile	16	18	16
Leu	24	28	24
Tyr	11	12	13
Phe	8	9	13
Lys	9	10	14
His	1	2	3
Arg	12	14	11
Trp	1	1	not determined
Total	224	244	231

2.2 Biological Properties of MMCs

α- and β-MMCs share similar amino acid composition with TCS, moreover, both their physical and chemical properties are also similar with each other, therefore, it is not unexpected that their biological activities also resemble those of TCS.

Both α- and β-MMCs possess abortifacient effect. When these two proteins are administered to pregnant mice, they induce mid-term abortion (Yeung *et al.,* 1985). This abortifacient effect is due to the deleterious effects of these proteins on the trophic activity of the visceral yolk sac and the syncytiotrophoblast of the chorioallantoic placenta (Chan *et al.,* 1986). Moreover, they can reduce the decidual response of the endometrium by selectively suppressing cellular proliferation and decidual cell formation (Chan *et al.,* 1986). Also, both MMCs can inhibit the uptake and incorporation of ^{3}H-leucine and ^{3}H-uridine into protein and RNA of the mouse blastocyst (Chan *et al.,* 1984). This inhibits the synthesis of macromolecules in the blastocyst and results in the retarded growth of the trophoblast and inner cell mass. The consequence is the failure in implantation and thus termination of early pregnancy.

Besides the abortifacient effect, α- and β-MMCs also inhibit some transformed trophoblasts cells such as malignant mole, choriocarcinoma and hydatidiform mole (Tsao *et al.,* 1986). Their actions are similar on both normal and abnormal cells. However, they

show no cytotoxic effect on cultured cells such as hepatoma and human fetal melanoma (Tsao *et al.,* 1986).

Both MMCs show suppressive effect on the cellular-mediated and humoral immune responses. They suppress the lymphocyte transformation and the proliferative response of some immune cells, including T-cells, B-cells and macrophages. Also, both MMCs have been shown to inhibit the replication of HIV in both acutely infected lymphoblastoid cells and chronically infected macrophages (Yeung *et al.*, 1986).

2.3 Ribosome Inactivating Activity of MMCs

In addition to the various aforementioned biological activities, α- and β-MMCs have also been reported to possess inhibitory effect on protein synthesis. They inhibit protein synthesis in the rabbit reticulocyte lysate system. This inhibitory effect on cell-free system takes place at nanomolar concentration of the MMCs (Yeung *et al.,* 1988). Consequently, both α- and β-MMCs can be classified as ribosome-inactivating proteins (RIPs). The enzymatic basis for the protein synthesis inhibitory effect of MMCs has been examined extensively. Like other plant RIPs, the MMCs act on the ribosome through their N-glycosidase activity (Poon, 1994). They release the diagnostic Endo's fragment from the 28S ribosomal subunit after acid aniline treatment. On the other hand, little or no N-glycosidase activity can be observed when the ribosomal proteins are first removed,

leaving the naked rRNA as substrate, indicating the importance of the ribosomal protein in the N-glycosidase activity. The N-glycosidase activity and the subsequent inhibition on protein synthesis may be responsible for some of the biological activities, for example, the abortifacient effect, of the MMCs. It is because protein synthesis is essential to embryogenesis, inhibition of the protein synthesis will result in retarded development of the embryo and uterus, and abortion is resulted.

2.4 Deoxyribonuclease Activity of MMCs

Besides the N-glycosidase activity, recently, α- and β-MMCs have also been reported to possess deoxyribonuclease (DNase) activity (Go, 1992; Go *et al.*, 1992). Under normal digestion conditions, they cleave the supercoiled, double-stranded SV-40 DNA to produce nicked circular and linear DNAs. However, prolonged incubation does not have any further effect. Moreover, the linear DNAs such as lambda, Ad-2 and T7 are not digested by the MMCs. Thus, it appears that the conformation of the DNA may be the determining factor for the DNase activity of these MMCs. Besides the MMCs, some other RIPs, for example, α-sarcin (Endo *et al.*, 1990) and trichosanthin (Li *et al.*, 1992) have also been reported to possess DNase activity.

Therefore, there are at least two different enzymatic activities in MMCs, namely N-glycosidase and DNase. As mentioned above, MMCs possess many different biological

activities which are believed to have an enzymatic basis. At the moment, it is still unknown how these biological activities are related to the two enzymatic activities observed and this is worth of further investigations.

3. Objectives of the Present Study

3.1 Rationale of the Study

At one time, it was believed that the N-glycosidase activity could account for all the biological effects of plant RIPs. It was also believed that the N-glycosidase activity is extremely specific and occurred only at single position in rRNA. Subsequent researches indicated that although this specific N-glycosidase activity, perhaps, is the most active one, however, it is by no means the only activity. Multiple depurination is demonstrated for some RIPs. Moreover, the substrate has also been extended from rRNA to mRNA, tRNA, polyA and even DNA, at least in saporin (Barbieri *et al.*, 1994). Besides the N-glycosidic cleavage, phosphodiester bond cleavage can also been detected with DNA as substrate. Thus, the enzymatic reaction of RIP is not as simple as once thought.

In the N-glycosidase activity, RIP can recognize RNA as substrate. In the DNase activity, RIP can cleave the phosphodiester bond. Therefore, it is probable that RIP can

cleave the phosphodiester bond in RNA. Moreover, such suggestion is also supported by the previous observation in the study of the MMCs' effect on naked rRNA (Poon, 1994). At high concentration, the MMCs acted on the naked rRNA to produce a RNA smear upon electrophoretic analysis. The observation is the same whether the sample is subsequently treated with acid aniline or not. Such results suggest that the MMCs might have a weak RNase activity. Such RNase activity was investigated in the present investigation.

3.2 Outline of the thesis

In the present investigation, the MMCs were purified by a simple procedure using two ion exchangers, namely, DEAE-cellulose and Mono-S FPLC column (Chapter 2). The ribonuclease activity was studied by both electrophoretic and spectrophotometric methods. Some commercially available RIPs were also used to investigate whether the RNase activity has a common occurrence in RIPs (Chapter 3). In addition to MMCs, the seeds of *Momordica charantia* also contain another much more potent RNase. It was purified and partially characterized (Chapter 4). In the final chapter, a conclusion will be given (Chapter 5).

Chapter 2 PURIFICATION OF MMCs

2.1 Introduction

RIPs can be found in many different types of plant. Even within the same plant, it is present in many different parts of the plant, including seed, leave, root and latices. The concentration of RIPs varies from one plant to another. It also varies among different tissues of the same plant. In general, a crude preparation of seed extract is usually more potent than other tissue of the same plant in ribosome inactivation (Barbieri *et al.*, 1993). Recently, the major attention on RIP study is focused on its application in the production of immunotoxin to act against cancer cells and anomalous growth cells. For example, the type I RIPs gelonin and momordin and the type II RIP ricin A-chain has been employed successfully for immunotoxin production (Lambert *et al.*, 1988).

To obtain a more efficient and powerful immunotoxin for therapeutic uses, it is necessary to purify more RIPs from different plant sources. An efficient purification procedure is also needed to obtain the RIPs for various enzymatic and biological studies. An ideal purification procedure should yield large amount of purified RIP in a relatively short period of time. However, the conventional method for RIP purification is by no means satisfactory. It involves many different steps, such as ion-exchange and gel filtration chromatographies. Between the chromatographies, it also need a number of dialyzing steps to remove the salt and lyophilization to concentrate the sample. These steps are quite time-consuming. Moreover, the application of acetone precipitation at the

beginning of the purification process might result in partial denaturation and decreased the final yield of the valuable toxin. Consequently, it is necessary to develop a more efficient purification scheme.

Like that of other RIPs, the purification of α- and β-MMCs from the seeds of *Momordica charantia* involves a number of time-consuming steps (Yeung *et al.*, 1985). Briefly, after acetone precipitation, the two MMCs are separated from each other by CM-Sepharose ion-exchange chromatography. For each of them, a gel filtration is needed to obtain the MMC in pure form (Fig. 2.1). These chromatographic steps, together with the associated dialysis and lyophilization, require at least a week to finish. As the time for the purification is very long, the MMCs might loss its enzymatic or biological activity. Therefore, it is desirable to improve the procedure so that it can be finished within a shorter time.

Affinity chromatography has been employed successfully in the purification of RIPs. The affinity ligand chosen is the triazine dyes which have been used in the isolation of several different types of enzyme, including kinases, dehydrogenases and some nucleotide-dependent enzymes (Dean *et al.*, 1979). Since the RIP uses nucleic acid as substrate, therefore, it is also expected they have some interaction with the triazine dye.

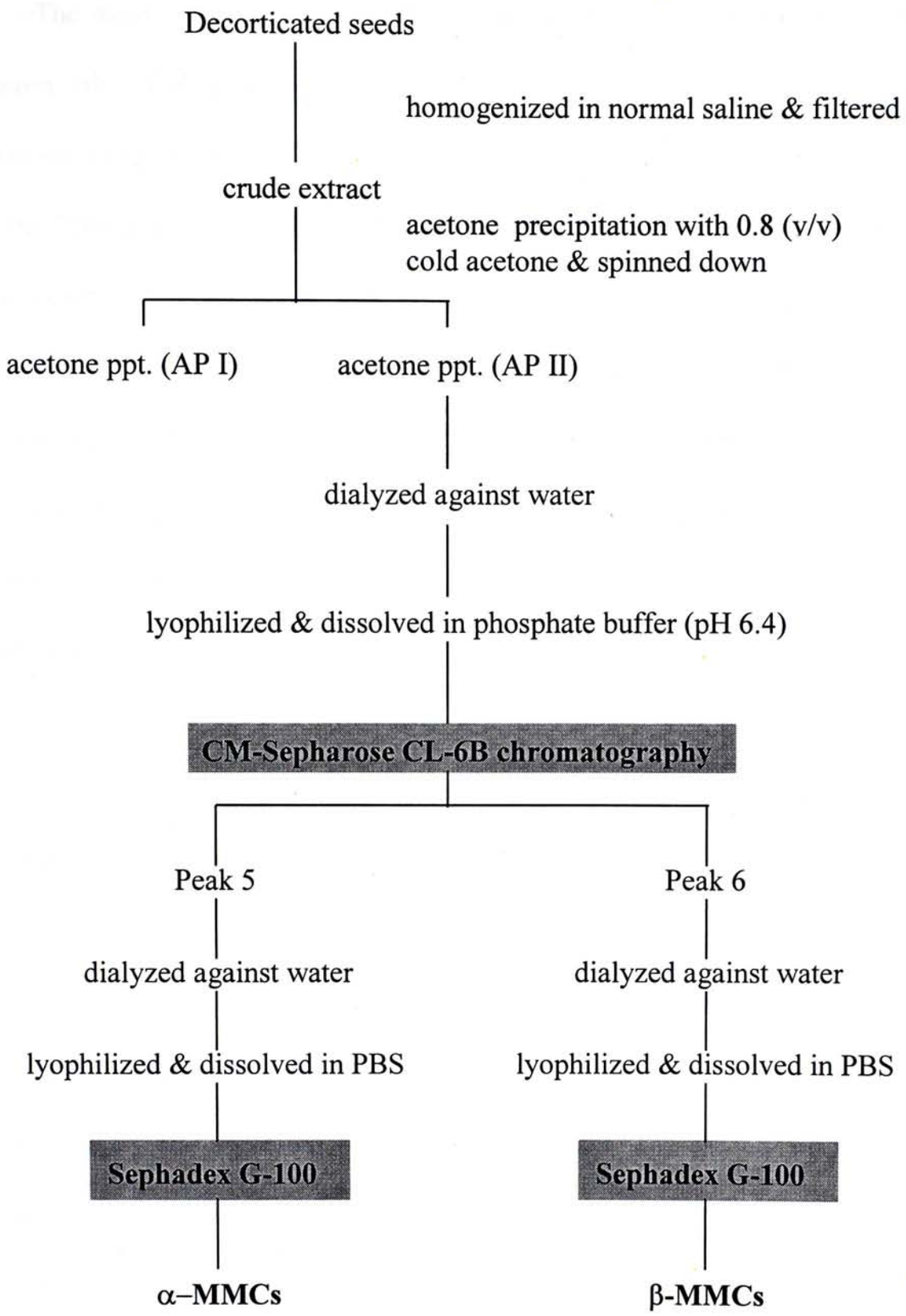

Fig. 2.1 Conventional purification procedure for α- and β-MMCs (Yeung *et al.*, 1985).

The most commonly used affinity resin is Affi-gel blue which is immobilized Cibacron Blue F3GA-agarose. The product of this dye is also available as Blue Sepharose. Cibacron Blue F3GA resembles the structure of dinucleotide and interacts with the RIPs at the active site of the protein. The retention capacity of the dye depends on the experimental conditions, such as pH, ionic strength and temperature as well as the nature and the concentration of the protein added (Munoz *et al.*, 1990). Besides being used for the purification of RIPs, the affinity ligand is also useful in the isolation of immunotoxin. The conjugated immunotoxin, because of the presence of the RIP moiety, will bind to the triazine dye whereas the unconjugated antibodies will be found in the breakthrough fractions and thus be separated (Cumber *et al.*, 1990).

The Affi-gel Blue affinity chromatography step is employed in a modified procedure for isolating α- and β- MMCs. It is used instead of acetone precipitation as the first step to isolate the RIPs from the crude seed extract (Go *et al.*, 1992) (Fig.2.2). The result is quite satisfactory as the Cibacron blue F3GA dye selectively interacts with the active site of the MMCs. Hence, most of the MMCs is bound onto the gel upon chromatography and separated from the crude extract. The MMCs are then eluted by sodium chloride solution. Besides this modification, the more efficient Mono-S FPLC ion exchange column is used instead of CM-Sepharose to separate the two MMCs. The resulting products are pure and there is no further need to pass each of the sample through the time-consuming gel filtration column. In short, this modified procedure involves only

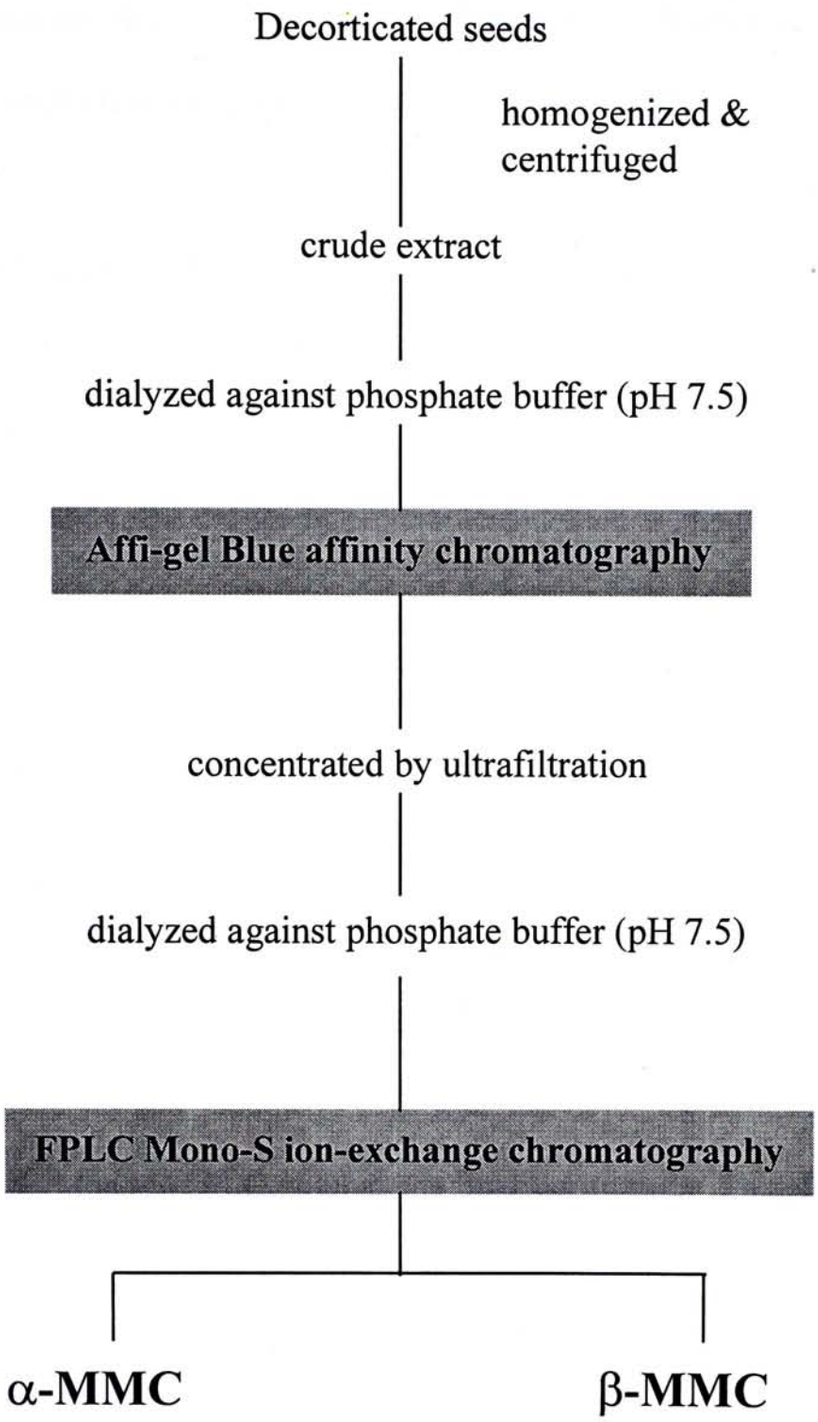

Fig. 2.2 Improved purification procedure for α- and β-MMCs (Go *et al.*, 1992).

two chromatographic steps, namely, Affi-gel Blue affinity chromatography and Mono-S FPLC ion-exchange chromatography.

The application of these two steps procedure reduces the purification time from one week to only two days, while the yield of both MMCs remains satisfactory. All the steps in the purification procedure are carried out at neutral pH and in aqueous environment, therefore, the chance of protein denaturation is significantly reduced. As a consequence, a larger amount of active proteins can be obtained from this new scheme.

Two years ago, the purification scheme for α- and β-MMCs was further modified (Poon, 1994) (Fig. 2.3). The efficient Affi-gel Blue affinity chromatography is replaced by a simple anion-exchange chromatography. The crude extract is first passed through the DEAE-cellulose column. The MMCs, being basic proteins, pass through the column unadsorbed. Since no salt elution is involved, therefore, the sample can be directly applied onto the Mono-S FPLC column for final purification. Therefore, considerable time could be saved. To sum up, this new scheme of purification involves two ion exchange steps, namely, DEAE-cellulose and Mono-S FPLC. This is also the scheme used for the purification of MMCs in the present study.

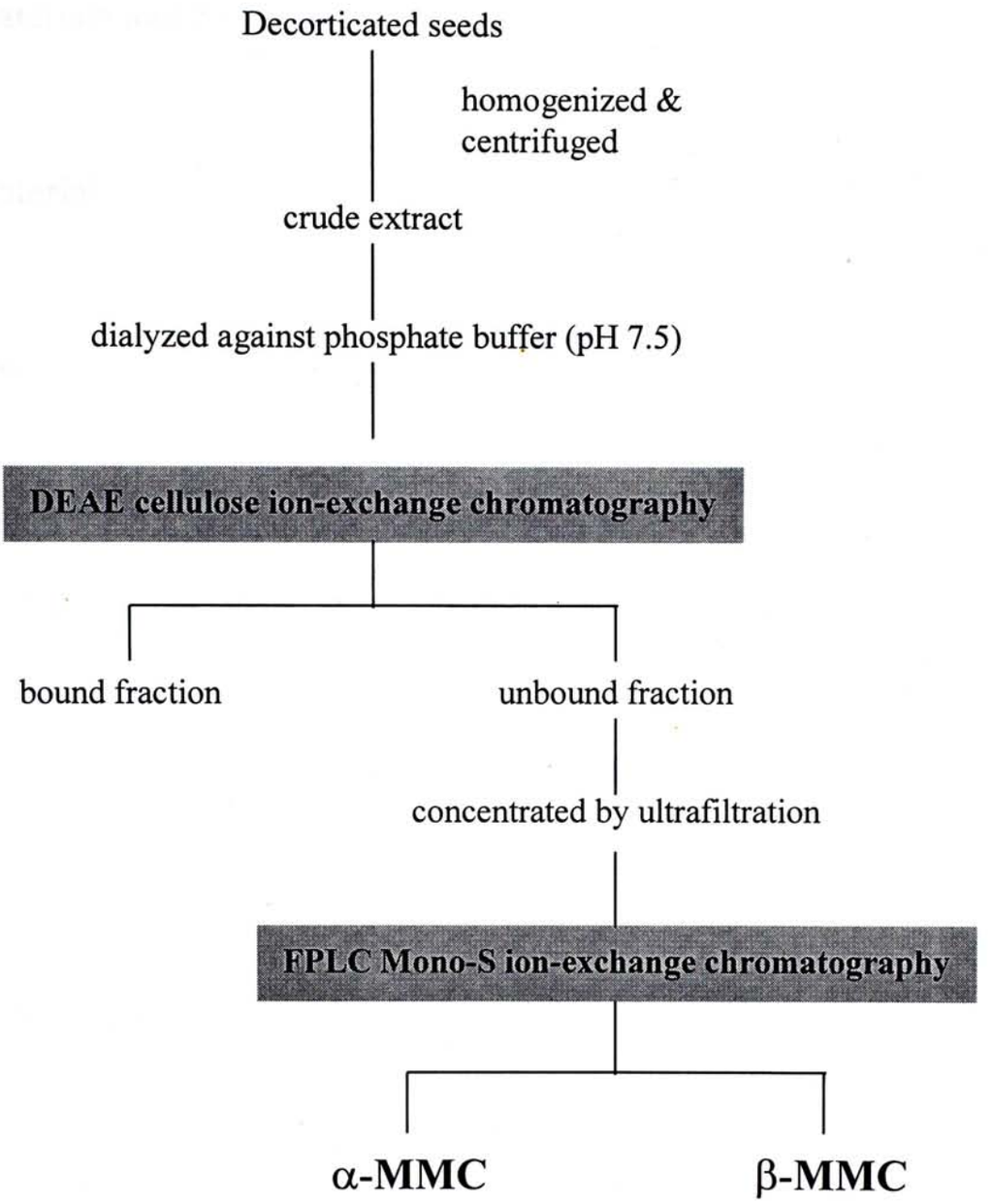

Fig. 2.3 The purification procedure used for isolating α- and β-MMCs in the present study (Poon, 1994).

2.2 Materials and Methods

2.2.1 Materials

Seeds of *Momordica charantia* (bitter gourd) were bought from the local market. DEAE cellulose (Fast Flow; Fibrous Form) was product of Sigma, Missouri, U.S.A. Mono S HR 5/5 FPLC column was purchased from Pharmacia LKB, Uppsala, Sweden. Reagents for electrophoresis were obtained from Bio-Rad, California, U.S.A. SPECTRA/POR® dialysis membrane (MWCO 10,000) was purchased from Spectrum, California, U.S.A. All other chemicals were of analytical grade and were used without further purification.

2.2.2 Purification of α- and β-MMCs

Ripe dried seeds of *Momordica charantia* (bitter gourd) were purchased from the local market. The seeds were stored in a cool dry place before use. In a typical preparation, decorticated seeds were ground in a mortar. 2.5g of the seed powder was added to 25ml of 2mM sodium phosphate, pH 7.5 and was homogenized by polytron (Kinematics). The slurry formed was stirred at 4°C overnight and then clarified by centrifugation (Beckman model J2-21) at 30,000*g* for 1 hour at 4°C. The pellet was discarded. The supernate was passed through two layers of cheesecloth to remove the

large particles. Then the filtered supernate was dialyzed in 2 litres of 2mM sodium phosphate, pH 7.5 overnight at 4°C with two changes of the buffer.

The dialyzed sample was applied to a DEAE-cellulose column (Econo-Column, 2.6 x 17.5 cm) pre-equilibrated with 2mM sodium phosphate, pH 7.5. After washing the column with the same buffer, the unbound fraction was collected and pooled together. The pooled fraction was concentrated by ultrafiltration (Amicon, PM 10 membrane). The concentrated sample was filtered through a 0.22μm filter before applying to a Mono-S FPLC column which has been equilibrated with 2mM sodium phosphate, pH 7.5. The proteins were eluted by a linear gradient of 0-60mM sodium chloride in 2mM sodium phosphate, pH 7.5 at a flow rate of 1ml/min.

The purified proteins were pooled accordingly and concentrated by ultrafiltration (Amicon, PM 10 membrane) to a protein concentration between 2 and 5mg/ml. It was then dialyzed against 2mM sodium phosphate, pH 7.5. The final concentrated proteins were aliquoted and stored at 4°C in sealed, autoclaved microfuge tubes (Eppendorf).

2.2.3 Protein Determination

Protein concentration was determined by the method of Lowry *et al.*, (1951) using bovine serum albumin (BSA) as standard. To each 0.2ml samples, 2ml solution A (1% w/v $CuSO_4 \cdot 5H_2O$: 2% w/v K,Na-tartrate : 2% w/v Na_2CO_3 in 0.1N NaOH, 1:1:100) was added. After mixing, the sample was allowed to stand at room temperature for 10min. before 0.2ml solution B (1 to 1 dilution of Folin's reagent in water) was added and mixed immediately. The sample was allowed to stand at room temperature for 45min. before the absorbance at 750nm was measured (Hitachi U2000 spectrophotometer).

2.2.4 Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis (SDS-PAGE)

Electrophoresis was performed according to the procedure of Laemmli and Favre (1973), using a 12% resolving gel and a 5% stacking gel. The gel was casted on the Mini-Protean set II (Bio-Rad). Samples were diluted with the sample loading buffer (10% glycerol, 0.4% SDS, 0.005% bromophenol blue and 20mM EDTA in 0.5M Tris-Cl, pH 7.5) followed by the addition of β-mercaptoethanol (5% v/v). The samples were boiled in water bath for 10min. Electrophoresis was performed at a constant current of 10mA at room temperature. After the tracking dye, bromophenol blue, reached the bottom of the gel, electrophoresis was stopped. The gel was stained with 0.115% Coomassie Brilliant

Blue R-250 in 8% (v/v) acetic acid, 25% (v/v) ethanol for 1 hour. The gel was then destained in acetic acid : ethanol : water (1:3:4) until the background is clear.

2.3 Results

After passing the clarified crude homogenate through the DEAE-cellulose column (Fig. 2.4), 86.6% of the soluble proteins in the crude extract was removed, leaving 13.4% of the soluble proteins in the unbound fraction (DEAE I) (Table 2.1). The unbound fraction contained 35.8mg of protein. SDS-PAGE analysis showed a major band at 30kDa which presumably, was the MMCs, together with some low molecular weight contaminants near the bottom of the gel (Fig. 2.5). The DEAE-I was then subjected to the FPLC Mono-S column for further purification.

Upon elution with a linear sodium chloride gradient of 0-60mM, DEAE-I was resolved into four major peaks on the Mono-S FPLC column (Fig. 2.6). These four peaks had the retention times of 25.51, 43.78, 47.24 and 67.58min., corresponding to sodium chloride concentrations of 14.7, 32.7, 36.7 and 56mM, and were named as peak I, II, III, and IV respectively. SDS-PAGE analysis suggested that peak I and peak II were small protein/peptides with M.W. ranging from 4 to 8kDa (data not shown). On the other hand, peak III and peak IV showed virtually only a single band of M.W. about 30kDa (Fig. 2.5). They were identified to be α-MMC and β-MMC respectively, as they had the same retention time as the α- and β-MMC purified by the conventional procedure (Yeung *et al.*, 1985). Consequently, both α- and β-MMCs were successfully purified from the crude

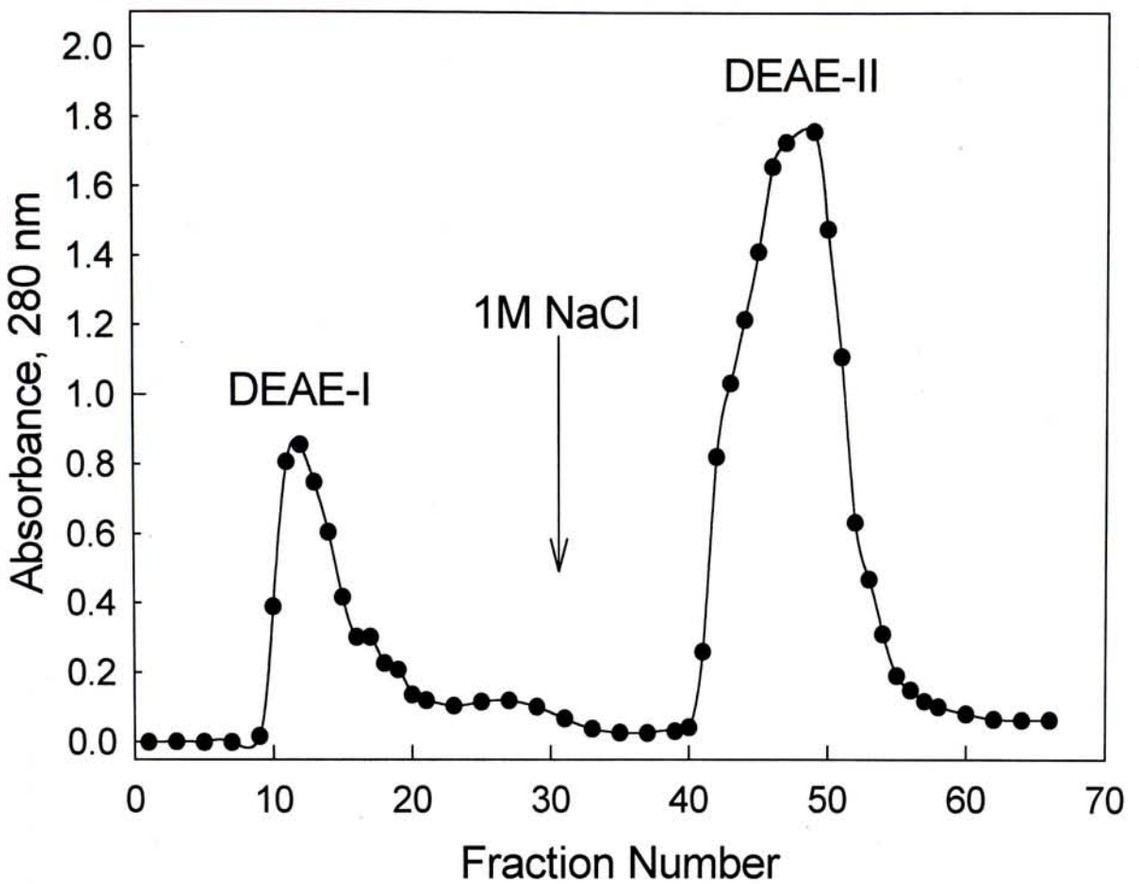

Fig. 2.4 Chromatography of the crude extract on DEAE-cellulose. The 30,000*g* supernate of seed homogenate, after dialysis against 2mM sodium phosphate, pH 7.5, was loaded onto a DEAE-cellulose column (2.6 x 17.5 cm) equilibrated with the dialyzing buffer. The column was washed with the dialyzing buffer and then eluted with 1M sodium chloride in the buffer. Fractions of 5.5ml were collected.

Table 2.1 Purification of α- and β-MMCs. The purification procedures are described in Section 2.2.2. Amount of protein was determined by the method of Lowry as mentioned in Section 2.2.3.

Purification Step	Amount of Protein (mg)
Crude extract	267
DEAE-I	35.8
FPLC Mono-S: α-MMC	3
β-MMC	0.7

* 2.5g decorticated seeds of *Momordica charantia* were used.

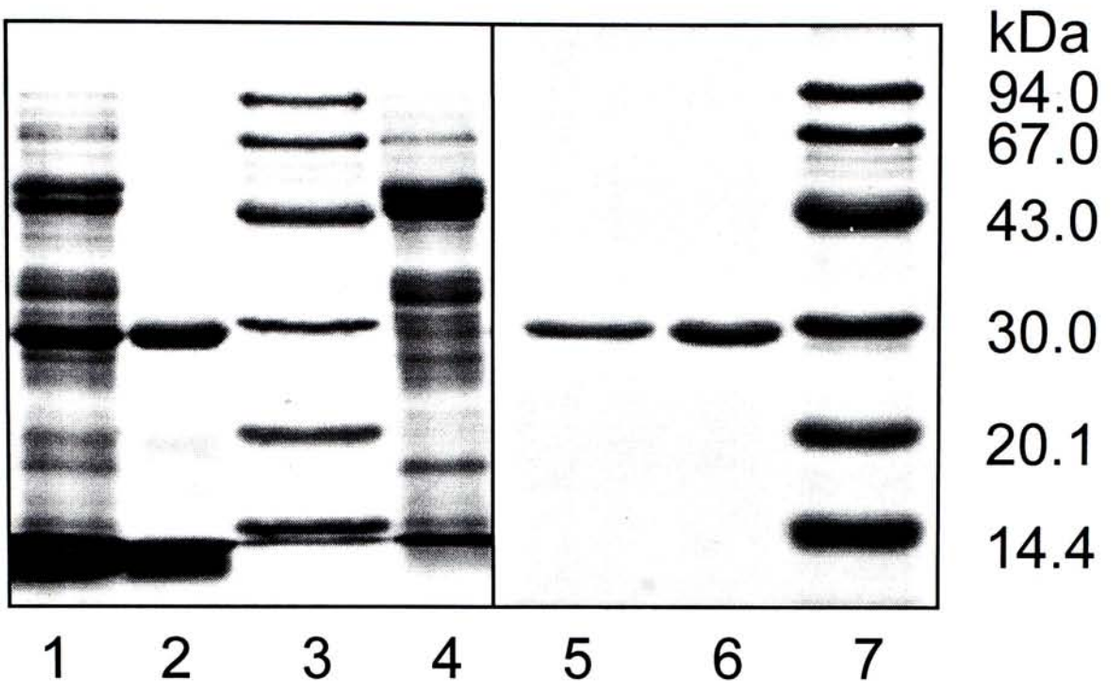

Fig. 2.5 SDS-PAGE of the sample at different stages of purification. Lane 1: 30,000*g* supernate of seed homogenate; lane 2: DEAE-I; lane 3 & 7: molecular weight standards (Pharmacia) including phosphorylase b (94.0kDa), bovine serum albumin (67.0kDa), ovalbumin (43.0kDa), carbonic anhydrase (30.0kDa), soybean trypsin inhibitor (20.1kDa) and α-lactalbumin (14.4kDa); lane 4: DEAE II; lane 5: purified α-MMC (Peak III from Mono-S column); lane 6: purified β-MMC (Peak IV from Mono-S column).

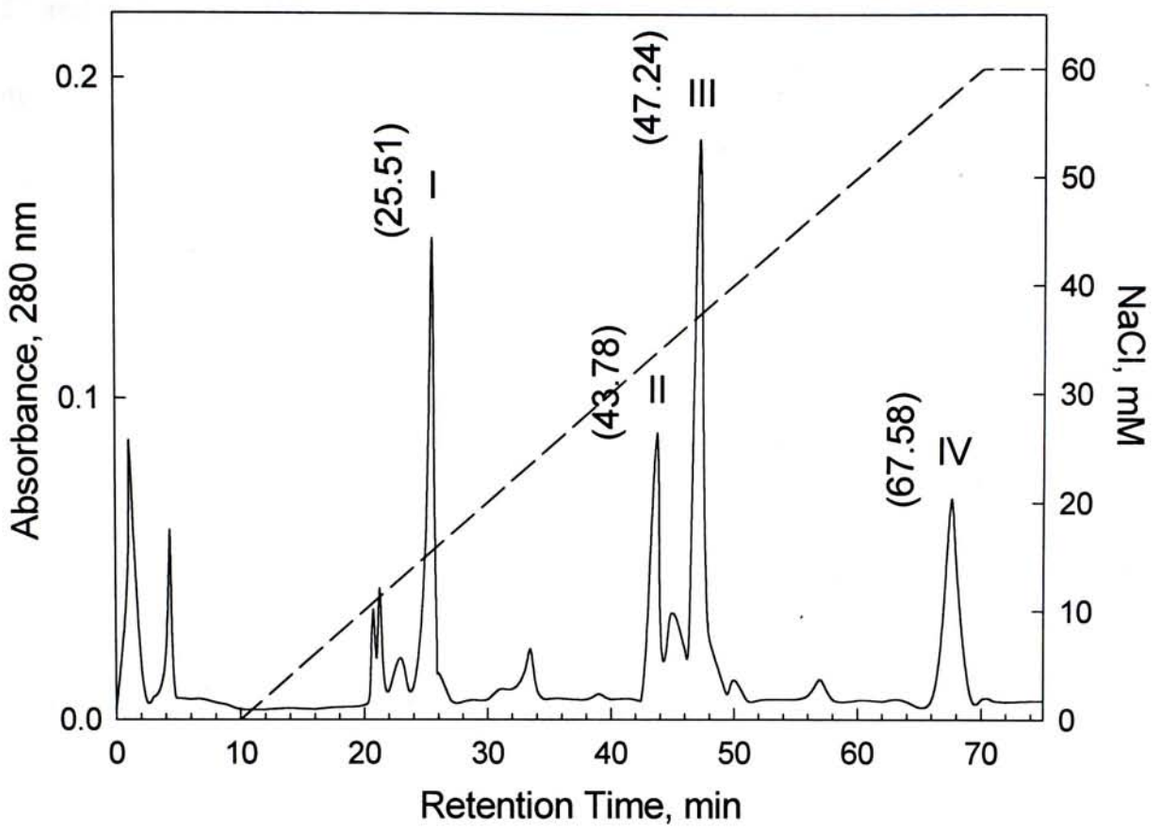

Fig. 2.6 Chromatography of DEAE-I on Mono-S FPLC column. DEAE-I, after concentration, was added to the Mono-S column and eluted with a linear gradient of 0-60mM sodium chloride in 2mM sodium phosphate, pH 7.5 at a flow rate of 1ml/min. Peaks III and IV were collected and identified as α- and β-MMCs respectively.

extract by using the present purification scheme. Using this scheme, about 3.0mg of α-MMC and 0.7mg β-MMC can be obtained from 2.5g of decorticated seeds in 2 days (Table 2.1).

2.4 Discussion

The purification scheme used for the isolation of α- and β-MMCs in this study consisted of two consecutive ion-exchange chromatographies. The first step is DEAE-cellulose anion-exchange chromatography while the second step involved the use of the Mono-S cation exchange FPLC column.

The use of the DEAE-cellulose as a first step of purification have several advantages. First, judging from the SDS-PAGE results, this step is quite efficient in removing the majority of the non-MMC proteins in the crude extract. Most of the non-MMC proteins in the crude extract are acidic in nature, therefore they bind onto the DEAE-cellulose and can be removed from the basic proteins, including the MMCs, which passed through the column in the breakthrough fraction. SDS-PAGE analysis also showed that DEAE-I only contain two molecular weight species. The 30kDa band represent the α- and β-MMCs, while the low molecular weight represents some contaminating proteins. Therefore, the DEAE-cellulose column is very useful and efficient in removing the non-MMC proteins from the seed extract. The removal of these non-MMCs proteins is essential for the success of the next purification step, since large amount of contaminating proteins may affect the resolution of the Mono-S FPLC chromatography.

Another advantage of using DEAE-cellulose is that the MMCs were unadsorbed by the column. In other words, they just passed through the column in the washing buffer. Such unadsorption shortens the time required for this chromatographic step. The unbound fraction can be collected easily and quickly. Moreover, the fact that no sodium chloride elution is needed also means that the time-consuming dialysis step before the second ion exchange Mono-S FPLC column can be eliminated. The only step needed is a concentration of the sample which can be achieved efficiently by ultrafiltration. This can further reduce the time for the purification process. In this respect, it is better than Affi-gel Blue. The MMCs adsorb to the Affi-gel Blue column and have to be eluted with 0.5M sodium chloride (Go *et al.*, 1992). Therefore, it is necessary to remove the salt before the next ion exchange step.

A third advantage of DEAE-cellulose is that it is relatively inexpensive. This is especially important when a large-scale preparation of MMCs is to be performed. In addition, DEAE-cellulose can be re-used easily after each purification, simply by washing the column with sodium chloride solution. On the contrary, Affi-gel Blue is much more expensive and may not be suitable for large scale use. Moreover, when seed extract is applied to the Affi-gel Blue column, some small solid particles may be trapped into the gel. Even after extensive washing with sodium chloride solution and buffer, these small particles retain in the column. After the gel has been used for several times, these small particles may block the column and affect the flow rate of the column. The binding

capacity of the column will also be affected. These contaminants may also cause a problem in preserving the gel, as they may lead to bacterial or fungal growth in the gel.

As a result of the above considerations, DEAE-cellulose is more efficient and cost-effective to use compared with Affi-gel Blue in the first step of purification process. Subsequent separation of the two MMCs and the removal of the final traces of contaminants can be easily achieved by the Mono-S FPLC column. Such simple, two-step procedure, both involving ion exchange chromatographies, affords milligram quantities of α- and β-MMCs in two days. Such a short time also reduce the chance of protein denaturation during the process.

Chapter 3 RIBONUCLEASE ACTIVITY OF MMCs

3.1 Introduction

RIPs are named because of their inhibitory effect on eukaryotic ribosome. Two different enzymatic mechanisms have been identified. In general, most plant and bacterial RIPs exert their effect on ribosome by the well studied N-glycosidase activity, however, fungal RIPs inhibit ribosome by virtue of their RNase activity. One exception is the recently identified luffin-S. Luffin-S is a plant RIP, however it differs from the typical RIP in that it has a much smaller molecular weight of about 10kDa. Of particular interest is that this plant RIP acts like the fungal RIP and possess the RNase activity to inhibit the ribosome (Gao *et al.*, 1994). This together with the recent finding that saporin can depurinate other RNA species and even DNA (Barbieri *et al.*, 1994) raise the question on the actual biological function of RIP in plant.

RIPs are frequently found at a high concentration in plant. Although it has not been detected in some plants, however, this may be due to the low sensitivity of the screening system used (Barbieri *et al.*, 1993). It is believed that RIP has a common occurrence in plants, and perhaps, its existence is ubiquitous in plants. To account for its wide existence, RIPs must have some important biological functions in plant. So far, several hypotheses have been formulated to explain its function (Barbieri *et al.*, 1993).

The RIPs may play a defensive role against predators. This seems possible only in the case of type II RIPs and in the few cases of type I RIPs which are present at a very high concentration. Nevertheless, these cases are few and cannot account for the widespread occurrence of RIPs. The fact that RIPs are usually more concentrated in seeds suggests that they may have certain function during germination. However, besides the concentration consideration, no further evidence is available to support such hypothesis. RIPs may also play a role in the defense mechanism against plant pathogens or viral infection. RIPs can effectively inactivate the ribosome at low concentration, therefore they can inhibit the replication of the foreign materials efficiently by inactivating their protein synthesis. RIPs may also play a metabolic role in the plant; however, the purpose of depurination of a specific RNA sequence remains unknown. Among these hypotheses, it appears that the defensive and metabolic function is the most likely natural role of RIPs in plant.

Recent findings show that saporin possesses unexpected multiple depurination activity on RNA species besides the ribosomal RNA (Barbieri *et al.*, 1993). Moreover, some RIPs also possess DNase activity (Go *et al.*, 1992; Li *et al.*, 1992). It is possible that the RIPs exert their effect not only on rRNA, but also on other RNA species and DNA through different enzymatic activities. RIP can recognize RNA and DNA as substrates in the N-glycosidase activity, it can also cleave the phosphodiester bond in DNA, however, whether it can cleave the phosphodiester bond in RNA remains un-explored.

In the present investigation, such RNase activity was examined, using α- and β-MMCs. It is necessary to investigate any possible activity of the MMCs in order to understand more about their natural roles. Moreover, MMCs possess many different biological activities, such as abortifacient effect, anti-tumor effect and the immunosuppressive effect (Yeung *et al.*, 1986). However, the exact enzymatic basis remains unknown. It is possible that some of these biological activities may not be related to the well-characterized N-glycosidase activity, but to some hitherto unknown activities. Consequently, the RNase activity of the MMCs are worth investigating.

3.2 Materials and Methods

3.2.1 Materials

α- and β-Momorcharins were purified from the seeds of *Momordica charantia* by ion exchange chromatographies on DEAE-cellulose and Mono-S FPLC column, as described in Chapter 2. Rabbit reticulocyte lysate (untreated) was purchased from Promega, Wisconsin, U.S.A. Aniline was from Merck & Co. Inc., New Jersey, U.S.A. Aniline (b.p. 184-186°C) was distilled prior to use. Since aniline is hydroscopic, the distilled aniline was stored in a dessicator before use. If aniline absorbs water, it would become yellow in color and another distillation is performed. Formamide and the dyes used in gel electrophoresis including ethidium bromide, bromophenol blue and xylene cyanol were purchased from Sigma. Agarose (high strength analytical grade) and the regents used for gel electrophoresis were from Bio-Rad. Photographic instant film model 667 was purchased from Polaroid, Massachusetts, U.S.A. DEAE-Sepharose and Mono Q HR 5/5 FPLC column were products of Pharmacia Biotechnology. PEI-cellulose TLC plate (10 x 10cm), saporin, tRNA, polyA, polyC, polyG and polyU were products of Sigma. For the experiments involving RNA, all the buffers were pre-treated with 0.1% (v/v) diethylpyrocarbonate (DEPC) for 18 hours at room temperature and then autoclaved to remove any traces of the chemical. All other reagents were of analytical or molecular biology grade and were used without further purification.

3.2.2 Precautions for Working with RNA

Since the major source of RNase contamination is our hands, gloves were worn at all times. Also, RNase contamination can come from bacteria and molds that may be present on airborne dust particles. Therefore, proper microbiological sterile technique was carried out when handling the reagents used for RNA works. Sterile, disposable plasticware was used for handling RNA as these materials are generally RNase-free and do not require pre-treatment. Whenever non-disposable glassware was used, it was baked overnight at 180°C before use. On the other hand, the non-disposable plasticware was first thoroughly soaked in a 0.1% (v/v) solution of DEPC, followed by extensive rinsing with sterile double-distilled water prior to use. In addition, water and all the buffer solutions were treated with 0.1% (v/v) DEPC, stirred overnight at room temperature and then autoclaved to remove any trace of DEPC. When Tris buffer was used, the solutions were not treated with DEPC directly since DEPC would react with the primary amine. Therefore, those solutions were prepared first with DEPC-treated water and then sterilized by autoclaving or filtering through a 0.22μm filter.

3.2.3 RNA Extraction and Recovery

Phenol was used to denature the protein and extract them out of an aqueous solution of RNA into the organic phase. Equal volume of phenol (containing 0.2% v/v β-mercaptoethanol) equilibrated with 0.1M Tris-Cl, pH 8.0, was added to the samples. The solution was then mixed gently until an emulsion was formed. The aqueous layer was separated from phenol by centrifugation. The aqueous layer was re-extracted with an equal volume of phenol : chloroform : isoamyl alcohol (25:24:1) for two times. Finally, an equal volume of chloroform : isoamyl alcohol (24:1) was utilized to remove the final trace amount of protein.

Two volumes of cold (-20°C), absolute ethanol and 0.1 volume of 3M sodium acetate (pH 5.2) were added to the RNA samples. After standing at -70°C for 1 hour, RNA was pelleted by centrifugation at 12,000*g* for 15min. at 4°C in a microcentrifuge. The pellet was washed with 70% ice-cold ethanol and recovered by centrifugation at 12,000*g* for 15min. at 4°C. The RNA pellet was dried by vacuum centrifugation (Speed Vac; Savant Instrument Inc., U.S.A.) and dissolved in appropriate buffer.

3.2.4 Activity towards rRNA

The N-glycosidase activity of the MMC toward rRNA was determined as described by Endo and his co-workers (Endo *et al.*, 1988). α- and β-MMCs was added to 25μl rabbit reticulocyte lysate with buffer A (25mM Tris-Cl, pH 7.6, 25mM KCl, 5mM $MgCl_2$) in a total volume of 70μl to give a final concentration of 0.3nM or as stated in the figure. The reaction mixture was then incubated for 30min. at 37°C. The reaction was stopped by chilling on ice and SDS was added to a final concentration of 0.5%. The total RNA was then extracted from the rabbit reticulocyte lysate by phenol/chloroform extraction and ethanol precipitation as described in Section 3.2.3. After the RNA pellet was dried under vacuum, the pellet was dissolved in 6μl of DEPC-treated water.

MMC-treated RNA was mixed with 60μl of 1M aniline/2.8M acetic acid (pH 4.5). After incubation for 3min. at 60°C, the mixture was chilled on ice and then spinned down for a few seconds. Then 0.1 volume (6.6μl) of sodium acetate (3M, pH 5.2) was added to the mixture. Finally, 1ml cold absolute ethanol was added and then precipitation was carried out at -70°C overnight. The precipitated RNA was recovered as described in Section 3.2.3. The aniline-treated RNA was then dissolved in 10μl DEPC-treated water and then analyzed electrophoretically.

3.2.5 Formamide Gel Electrophoresis

An aliquot of 3μl precipitated RNA was mixed with 1μl of electrode buffer (36mM Tris-Cl, 30mM $Na_2H_2PO_4 \cdot 2H_2O$, 2mM EDTA·$Na_2 \cdot 2H_2O$) and 6μl 100% formamide. The mixture was then incubated for 5min. at 65°C. After incubation, the sample mixture was chilled on ice and spinned down for a few seconds. Later, 1μl of 11x gel loading buffer (0.5M EDTA, pH 8.0 and 0.5% w/v bromophenol blue in glycerol) was added to the sample mixture.

Formamide gel (1.2%) was prepared by melting 0.36g agarose in 12ml DEPC-water in a microwave oven. Then 3ml of electrode buffer and 15ml 100% formamide were added to the melted agarose solution and mixed well. After cooling for several min., the solution was poured into a plastic mould. After the gel is set, the formamide gel is ready for RNA analysis.

The sample mixture was applied to the sample well and gel electrophoresis was carried out at a constant voltage of 50V for 1.5 hour in the electrode buffer (0.1X electrode buffer, 50% formamide). After electrophoresis, the gel was stained in ethidium bromide (0.5μg/ml) for 20min. with gentle shaking. Then the gel was washed with several changes of distilled water for 30min. and then photographed with the Polaroid 667 instant film.

3.2.6 Activity toward tRNA

The activity of MMCs and other RIPs toward tRNA was determined by measuring the production of acid-soluble UV absorbing species by a modification of the method of Kunitz 1946. The MMC (or saporin) was incubated with 200μg of tRNA in 150μl of 100mM sodium acetate, pH 5.0, at 37°C for 1 hour. For the study on the pH dependence of activity, three different buffer systems, namely, sodium acetate (pH 4-5.5), Mes (pH 5.5-7) and Hepes (pH 7-8) were used. The reaction was terminated by the addition of 350μl of ice-cold 3.4% perchloric acid. After 15min. standing on ice, the sample was centrifuged at 15,000*g* for 15min. at 4°C. The absorbance of the supernatant, after 10 fold dilution with DEPC-water, was measured at 260nm. One unit of enzyme activity is defined as the amount of enzyme that produces an absorbance increase at 260nm of one per min. in the acid soluble fraction per ml of reaction mixture under specified condition.

3.2.7 Activity toward Polyhomoribonucleotides

The activity toward the polyhomoribonucleotides was determined by a modification of the method of Zimmerman and Sandeen (1965). The MMC (or saporin) was incubated with 100μg of polyA, polyC, polyG or polyU in 250μl of 100mM sodium acetate, pH 5.0 at 37°C for 1 hour. The reaction was terminated by the addition of 250μl of ice-cold 1.2N perchloric acid with 20mM lanthanum nitrate. After 15min. standing on ice, the sample was centrifuged at 15,000*g* for 15min. at 4°C. The absorbance of the supernatant, after 10 fold dilution, was measured at 260nm (polyA, polyG and polyU) or 280nm (polyC).

3.2.8 Analysis of Reaction Product by DEAE-Sepharose

The reactions of β-MMC and saporin toward polyA and polyU were carried out as described in Section 3.2.7. The reaction was stopped by boiling at 100°C for 10min. The boiled reaction mixture was centrifuged and 100μl of the supernatant was loaded onto a 1ml syringe packed with 200μl of DEAE-Sepharose equilibrated with 40mM Tris-Cl, pH 8.0 (Fig. 3.1). The breakthrough fractions were collected and the total amount of UV absorbing species at 260nm was determined. The standards, including adenine, uracil, AMP, UMP, polyA and polyU, were also tested for their binding on the DEAE-Sepharose under the same condition.

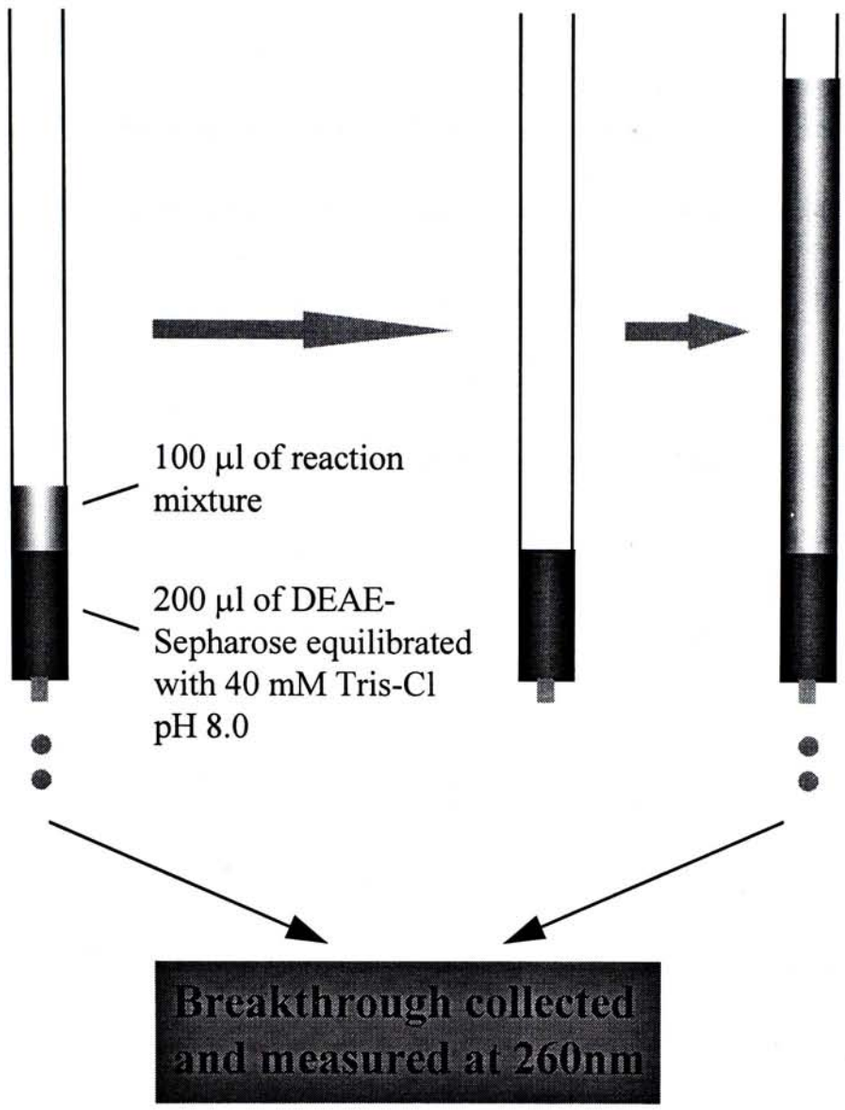

Fig. 3.1 Reaction product analysis for the action of β-MMC and saporin toward polyhomoribonucleotides by DEAE-Sepharose.

3.2.9 Analysis of Reaction Product by PEI-cellulose TLC Plate

Saporin was allowed to react with polyA as described in Section 3.2.7. The reaction was terminated by boiling the reaction mixture at 100°C for 10min. The boiled reaction mixture was then centrifuged, the supernatant was recovered and was ready for product analysis. PEI-cellulose TLC plate with fluorescent indicator (5 x 10cm) was used. The TLC was carried out by a modification of the method of Raaen (1968) and Randerath (1964). It was run under a gradient basis, five concentrations of LiCl (0.1M, 0.2M, 0.5M, 1M and 2M) was used. Five μl of the sample or standards were applied at the sample application line about 1cm above the bottom of the plate. After the spots were dried, the plate was soaked in 0.1M LiCl solution and TLC was run for 1cm above the sample application line. The plate was not allowed to dry before soaking into 0.2M LiCl solution and run for another 1cm. Such procedure was repeated for the other concentrations of LiCl solution (Fig. 3.2). Finally, the spots were visualized with a UV lamp (254nm) and photographed.

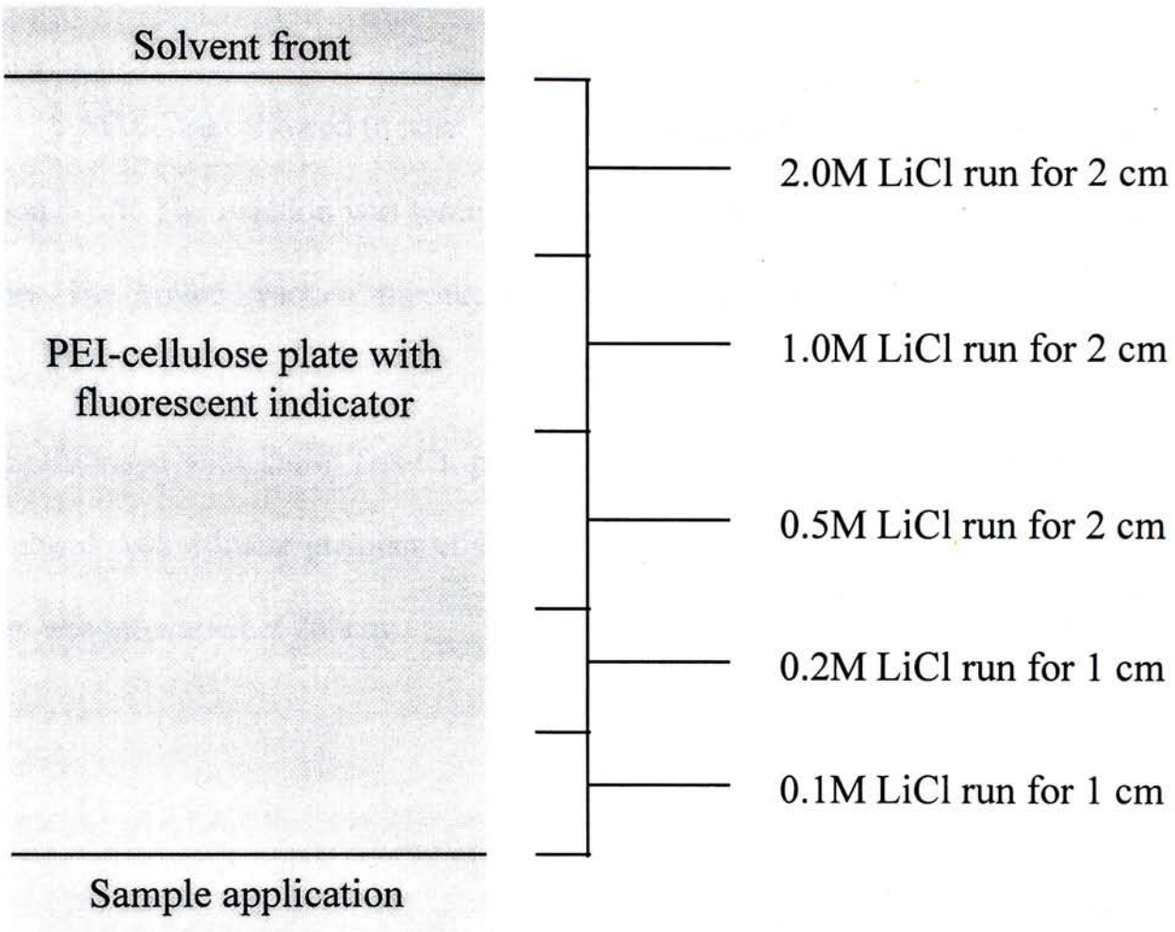

Fig. 3.2 Reaction Product Analysis of saporin toward polyA by PEI-cellulose TLC plate.

3.2.10 Analysis of Reaction Product by FPLC Mono-Q Chromatography

β-MMC was allowed to react with polyU under the reaction condition as stated in Section 3.2.7. The reaction was terminated by boiling the reaction mixture at 100°C for 10min. The boiled reaction mixture was then centrifuged and the supernatant was recovered for chromatographic analysis on the Mono-Q FPLC column. The column was pre-equilibrated with 2mM Tris-Cl, pH 8.0. After the sample was injected, the column was eluted with a linear gradient of 0-100mM sodium chloride in the same buffer. The eluent was monitored at 260nm.

3.3 Results

3.3.1 Activity of α- and β-MMCs toward rRNA

The electrophoretic pattern of the rRNA remained the same after the intact ribosome in rabbit reticulocyte lysate was treated with either α- or β-MMC. However, further treatment of the rRNA with aniline under acidic condition resulted in the generation of a specific RNA fragment of ~400 nucleotides. The position of the 28S rRNA was also shifted and its mobility became slightly faster than that of the control (Fig. 3.3). On the other hand, when the rRNA was first extracted from the ribosome and used as the substrate for the reaction, no specific new RNA fragment could be observed. Instead, electrophoretic analysis revealed a smear of RNA after the naked rRNA was treated with micromolar concentration of both MMCs. The same pattern was observed even when the MMC-treated rRNA sample had not been further treated with aniline (Fig. 3.4).

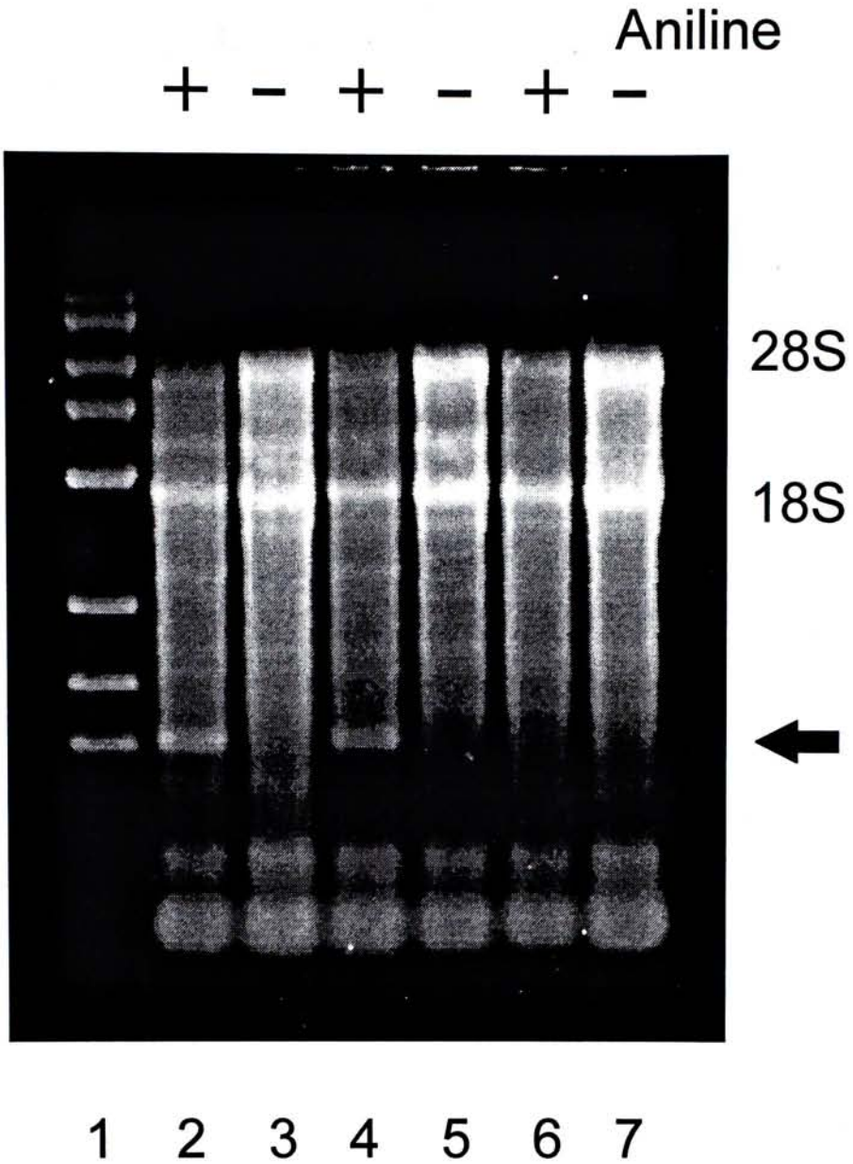

Fig. 3.3 Effect of α- and β-MMCs on intact rRNA. Rabbit reticulocyte lysates were incubated with MMC (0.3nM) in 25mM KCl, 5mM $MgCl_2$, 25mM Tris-Cl, pH 7.6 at 37°C for 30min. The rRNA was extracted and analyzed in 1.2% agarose gel containing 50% formamide. "+" and "-" indicate, respectively, the presence and absence of aniline treatment of the rRNA before electrophoretic analysis. The arrow denotes the new fragment of ~400 nucleotides produced by acid aniline treatment. Lane 1: RNA markers (Promega); lanes 2 & 3: α-MMC, lanes 4 & 5: β-MMC, lanes 6 & 7: control without MMC treatment.

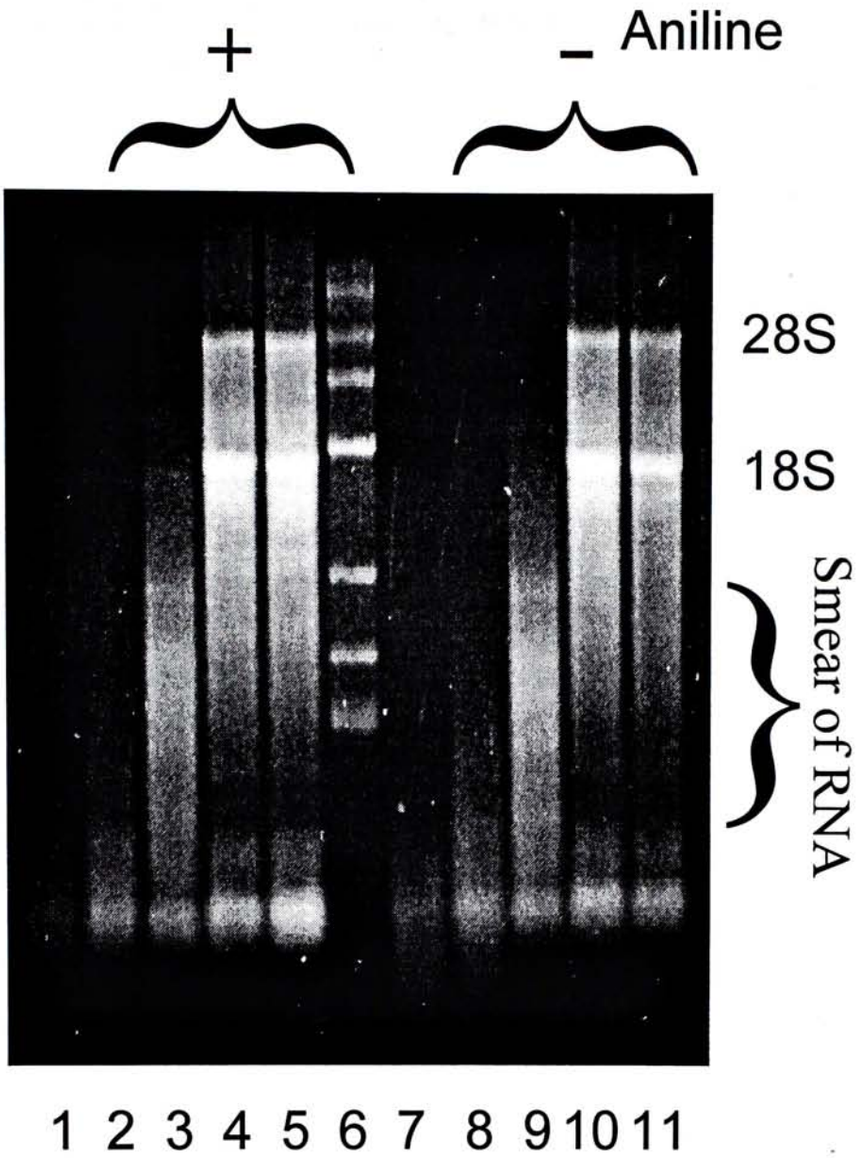

Fig. 3.4 Effect of α-MMC on naked rRNA. Naked rRNA was prepared from rabbit reticulocyte lysate by phenol/chloroform extraction. The naked rRNA was incubated with different concentration of α-MMC (lane 1-5: 10000, 1000, 100, 10, and 0nM respectively) in 25mM KCl, 5mM $MgCl_2$, 25mM Tris-Cl, pH 7.6 at 37°C for 30min. The rRNA was treated with acidic aniline and then analyzed in 1.2% agarose gel containing 50% formamide. Lane 6: RNA markers (Promega). Lanes 7-11: similar to lanes 1-5, except that the samples were not treated with acidic aniline before electrophoretic analysis.

3.3.2 Activity of α- and β-MMCs toward tRNA

Both MMCs were found to act catalytically on tRNA, as shown by the release of acid-soluble UV absorbing species. The activity varied with the pH of the reaction mixture. Maximal activity was observed at pH around 5.5 for β-MMC (Fig. 3.5). The activity of β-MMC was at least 10 fold higher than that of α-MMC throughout the entire pH range studied.

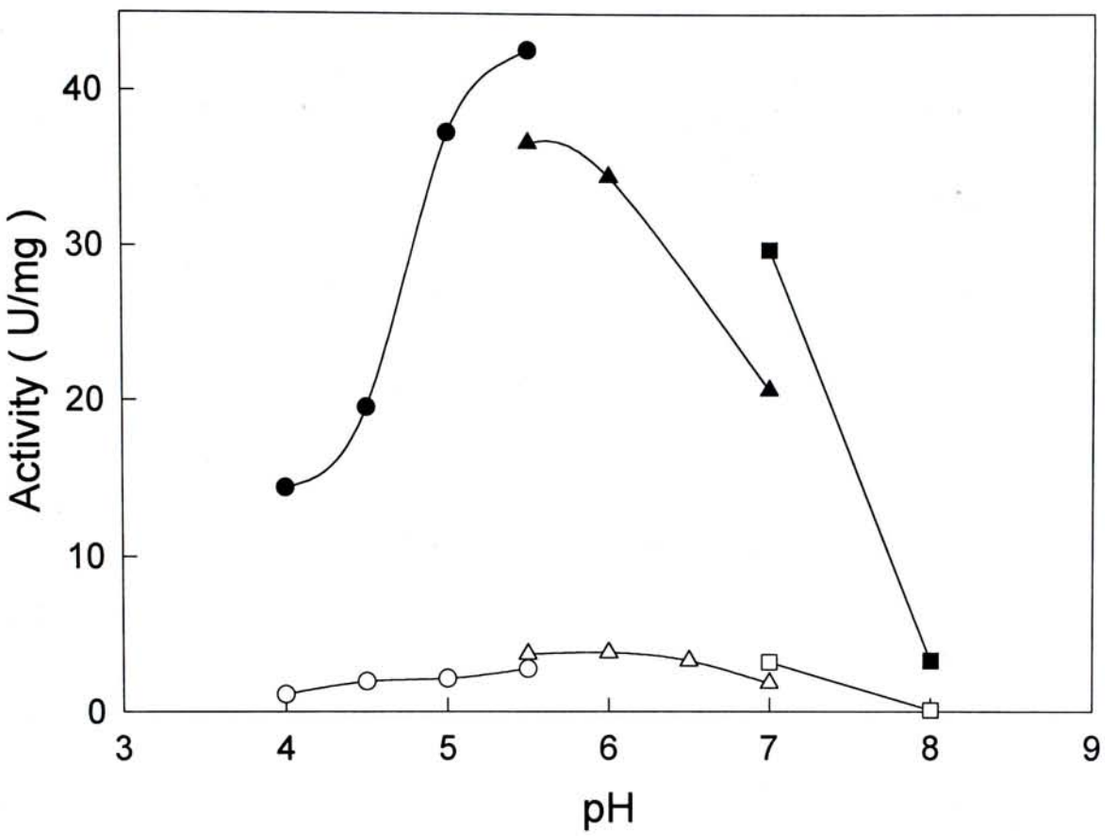

Fig. 3.5 Effect of pH on α-(open symbols) and β-(closed symbols) MMC's action on tRNA. The MMC was incubated with 200μg of tRNA in a final volume of 150μl 100mM sodium acetate (●), Mes (▲) or Hepes (■) at 37°C for 1 hour. The reaction was stopped by the addition of 350μl of ice-cold 3.4% perchloric acid. After standing on ice for 15min., the sample was centrifuged at 15,000*g* for 15min. at 4°C. The absorbance of the supernatant, after suitable dilution, was measured at 260nm.

3.3.3 Activity of α- and β-MMCs toward polyhomoribonucleotides

Although β-MMC had a much higher activity than α-MMC toward the polyhomoribonucleotides, the substrate specificity pattern was very similar. Both of them acted preferentially on polyU, however, their activity toward the other three polyhomoribonucleotides were negligible, or at least 10 fold lower than that on polyU (Table 3.1). No UV absorbing products can be detected in the DEAE breakthrough fractions after the reaction of β-MMC on polyA and polyU. Under the same condition, polyA, polyU, AMP and UMP were completely adsorbed on the anion exchanger whereas the free bases adenine and uracil were found in the breakthrough fractions (Table 3.2). Analysis of the reaction product of β-MMC on polyU, upon comparison with the standards (Fig. 3.6), indicated that the polyU was first cleaved into oligoribonucleotides of different sizes and finally to UMP, but no uracil was detected throughout the entire reaction process (Fig. 3.7). Prolonged incubation also failed to detect any uracil (data not shown).

Table 3.1 Activities of α-, β-MMC and saporin on various polyhomoribonucleotides[a].

	Activity (U/mg protein)		
Substrate	α-MMC	β-MMC	Saporin
polyA	2.34	9.16	82.79
polyC	<0.1	<0.1	<0.1
polyG	0.45	6.87	0.87
polyU	23.52	377.68	223.09

[a]The activity was determined as described in Section 3.2.6.

Table 3.2 Comparison of the amount of UV absorbing species after reaction using the precipitation assay and the DEAE analysis.

Reaction mixture	Total Absorbance[b] 260nm	
	acid soluble	DEAE breakthrough
(A) Reaction[a]		
β-MMC + polyA	0.05	0
β-MMC + polyU	2.29	0.047
saporin + polyA	1.69	1.71
saporin + polyU	1.81	0.011
(B) Standards		
A	+[c]	+
AMP	+	-
polyA	-	-
U	+	+
UMP	+	-
polyU	-	-

[a]The assays were performed as described in Section 3.2.7 and 3.2.8. The amount of β-MMC and saporin used were 2.33 and 12.24μg respectively.

[b]The total absorbance is obtained by multiplying the absorbance with the total volume (ml).

[c] "+" indicates the presence of >95% and "-" indicates the presence of <5% of the sample added.

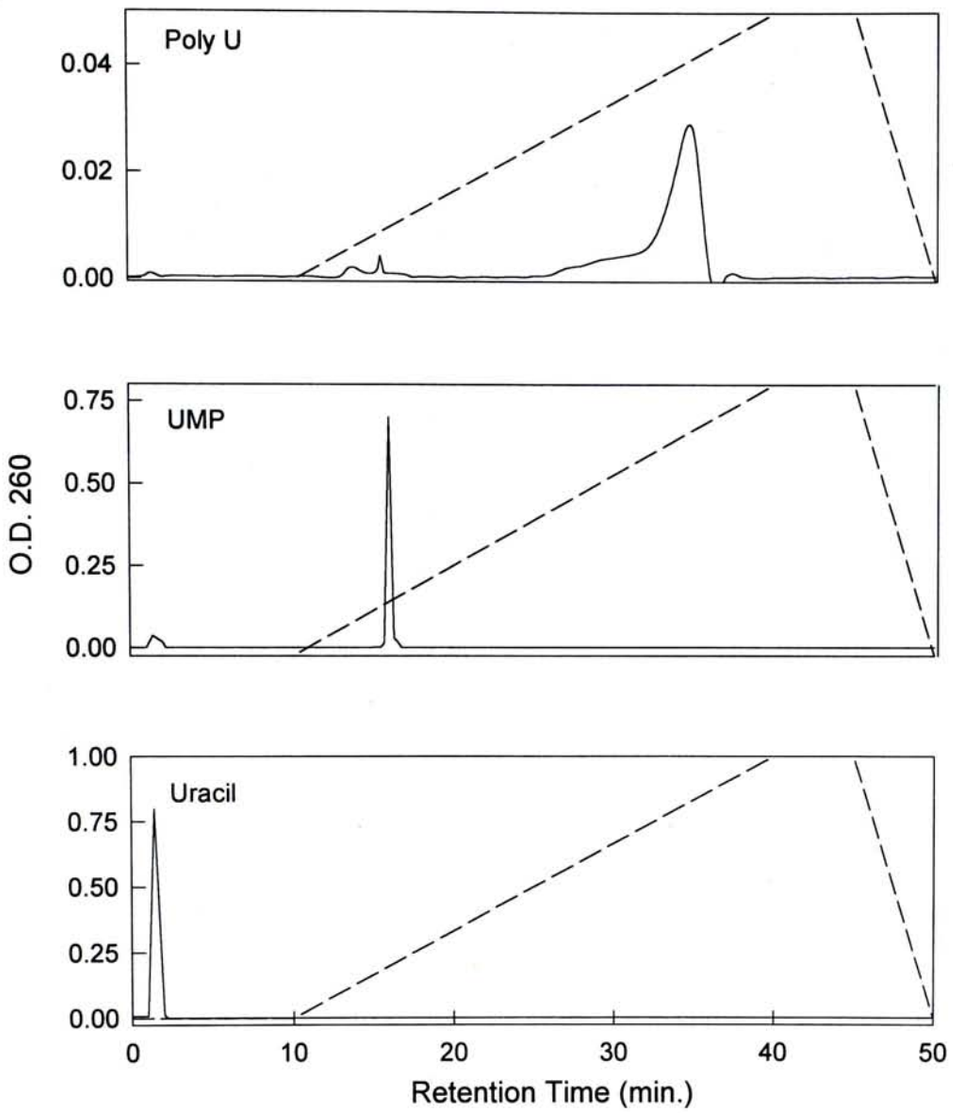

Fig. 3.6 Analysis of the standards by Mono-Q FPLC chromatography. The Mono-Q FPLC column was equilibrated with 2mM Tris-Cl buffer, pH 8.0. The standards, uracil, UMP and polyU were injected into the column. The system was run under a linear sodium chloride gradient form 0-100mM for 30min. The O.D. reading was recorded at 260nm during the chromatography.

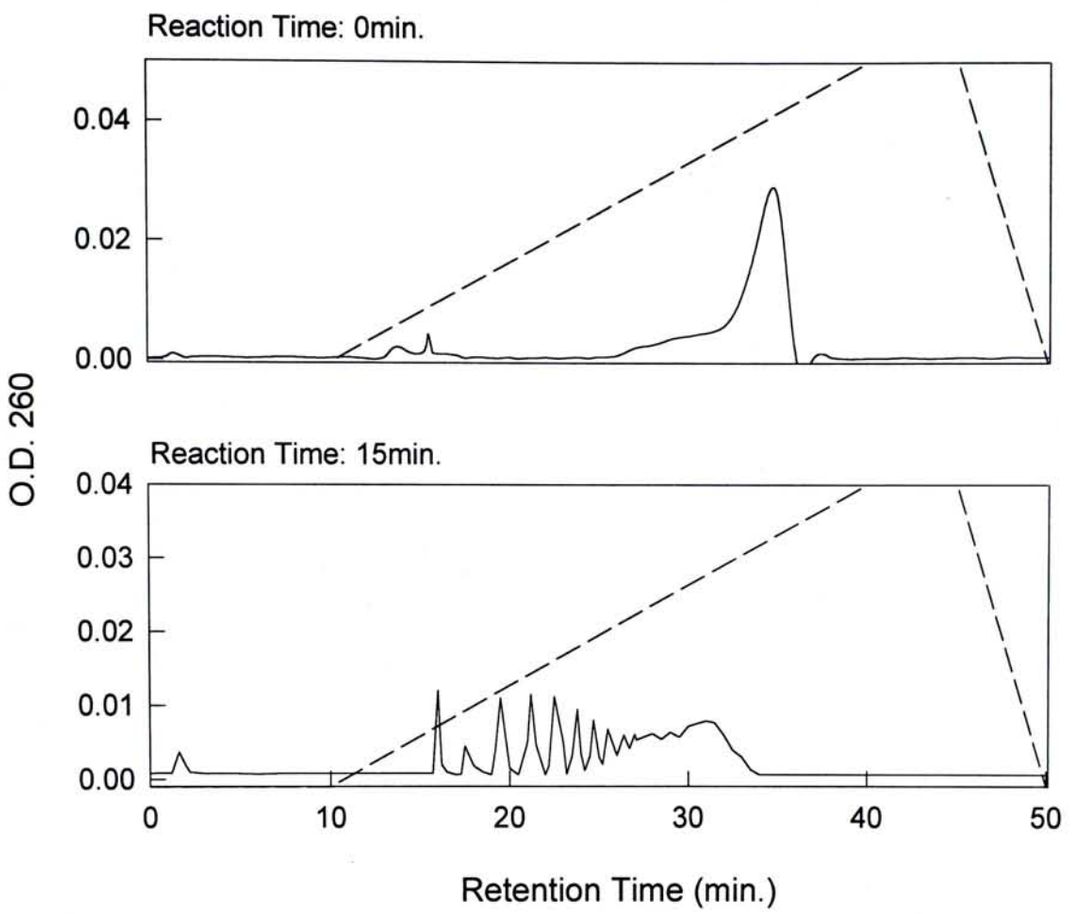

Fig. 3.7 Product analysis of the time course reaction between β-MMC and polyU by Mono-Q FPLC chromatography. The Mono-Q FPLC column was equilibrated with 2mM Tris-Cl buffer, pH 8.0. The reaction was performed as described in Section 3.2.7, the reaction was stopped at different time intervals by boiling the reaction mixture at 100°C for 10min. The sample was then centrifuged and injected in the Mono-Q column for analysis. The system was run under a linear sodium chloride gradient form 0-100mM for 30min. The O.D. reading was recorded at 260nm during the chromatography.

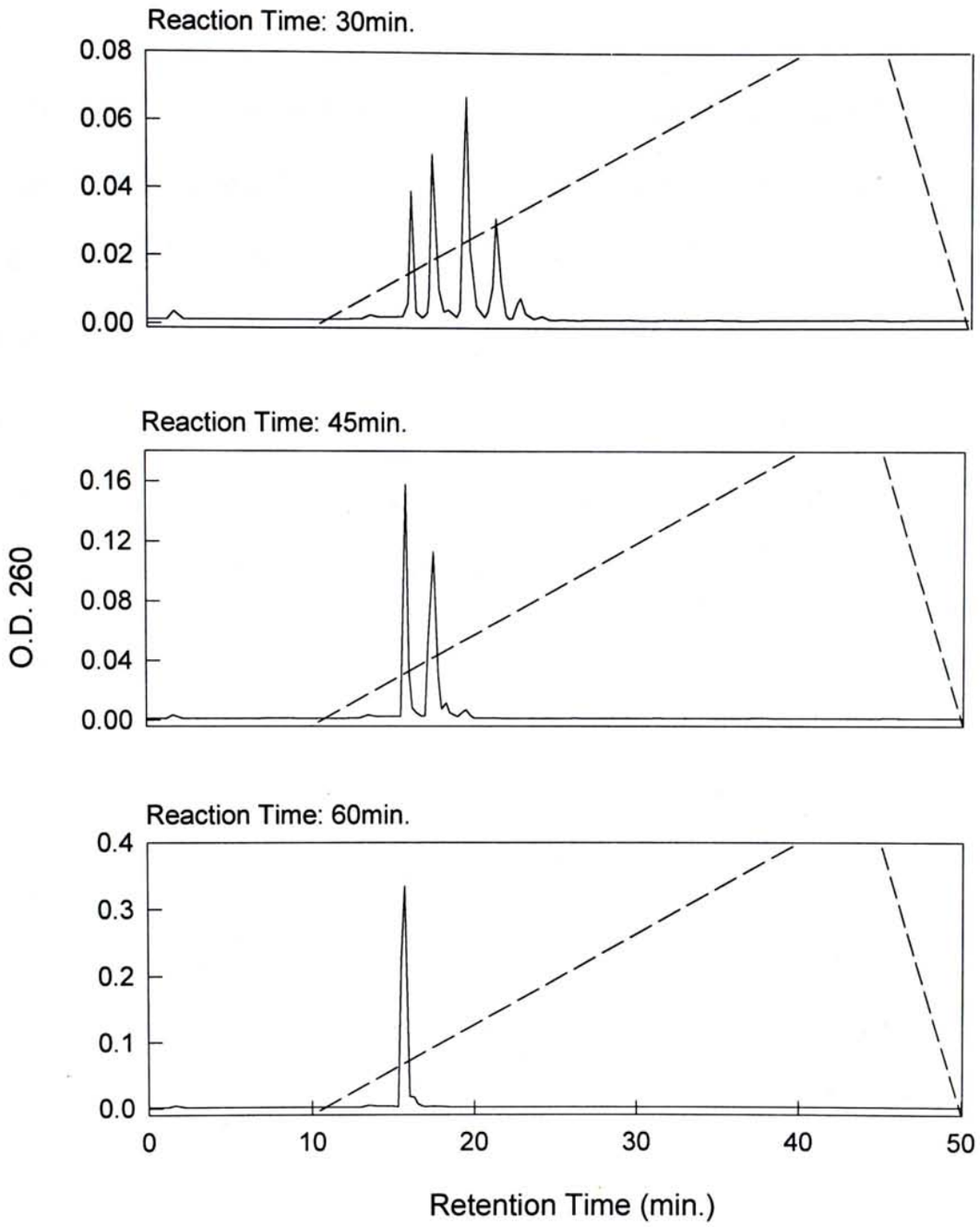

Fig. 3.7 Continued.

3.3.4 Activity of saporin on polyhomoribonucleotides

Saporin had a high activity on both polyA and polyU, but virtually no activity on polyC and polyG (Table 3.1). TLC analysis on PEI-cellulose plate showed that adenine was released from polyA after treated with saporin (Fig. 3.8), however, no AMP can be observed. DEAE chromatographic analysis of the product also indicated that all the UV absorbing species remained unadsorbed by the anion exchanger for the reaction on polyA. However, the reaction on polyU was different. With polyU as substrate, no UV absorbing species can be detected in the DEAE breakthrough fractions. (Table 3.2).

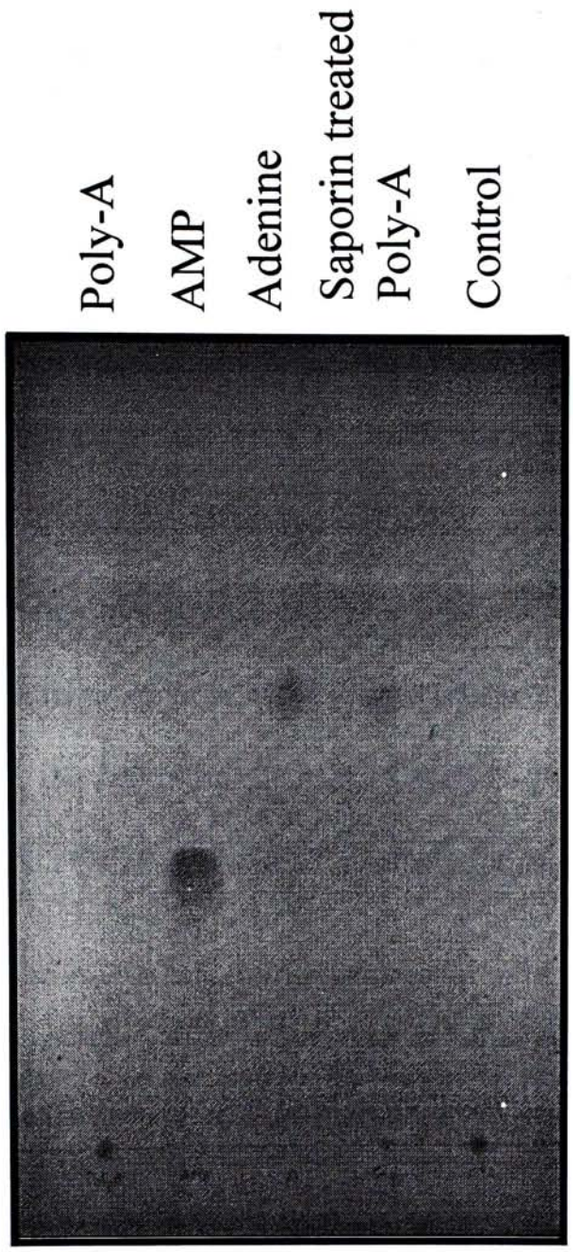

Fig. 3.8 Product analysis of the reaction between saporin and polyA by PEI-cellulose TLC plate. The reaction was carried out as described in Section 3.2.7. For the control, no saporin was added. The reaction was stopped by heating at 100°C for 10min. The reaction mixture was centrifuged and 5μl of the supernatant (control and test), together with the standards (polyA, AMP and adenine) were applied onto the plate. The plate was run following the procedures stated in Section 3.2.9.

3.4 Discussion

Both α- and β-MMCs acted on the ribosomal RNA, the action was very similar to each other. They reacted only with the 28S rRNA but not the 18S, 5.8S or 5S rRNA (Fig. 3.3). They released a specific Endo's fragment from the 28S ribosomal subunit after the acid aniline treatment. Since no such RNA band can be observed without acid aniline treatment, therefore, it is obvious that the reaction involved N-glycosidic cleavage. Moreover, as there were no other RNA bands generated after the reaction, it is believed that the N-glycosidase activity of both MMCs only released a single adenine molecule from the rRNA. Indeed, the action mechanism of such N-glycosidase activity has also been examined using the crystal structure of α-MMC (Huang *et al.*, 1995; Ren *et al.*, 1994).

Such N-glycosidase activity was very active and can be detected at nanomolar concentration of the MMC, using intact ribosome from rabbit reticulocyte lysate as substrate. However, when the ribosomal proteins were first removed from the incubation mixture, leaving the naked rRNA for reaction, the N-glycosidase activity was not apparent even at much higher enzyme concentration. Therefore, the presence of the ribosomal proteins is very important for the N-glycosidase activity to take place. Similar observation has been reported for the action of ricin A-chain (Endo *et al.*, 1988).

On the other hand, at a high concentration of MMCs, some non-specific cleavage on the rRNA occured. A smear was observed upon electrophoretic analysis and the addition of aniline did not make any difference in the pattern. This suggested that the cleavage reaction involved was ribonucleolytic, but not N-glycosidic, in nature. Such ribonuclease activity has been reported for ricin (Obrig *et al.*, 1985). However, it has not been studied in detail, perhaps as a result of its lower activity and lower specificity when compared with the N-glycosidase activity. The observation that only naked rRNA was cleaved suggested that the ribosomal proteins may protect the ribosome from the ribonucleolytic attack of MMCs. Therefore, intact ribosomal RNA is not be a suitable substrate for studying the RNase activity of the MMC, instead other RNA species, for example, tRNA and the polyhomoribonucleotides, were used as the reaction substrates.

Moreover, to simplify the experimental system for studying the action of MMCs on RNA, spectrophotometric analysis was used instead of the electrophoretic method, and tRNA was used instead of rRNA. The release of the acid-soluble UV absorbing products indicated that tRNA was a good substrate for the MMCs, especially for β-MMC. From the result of the pH profile, it was shown that maximal activity occurred at pH around 5.5. Moreover, the activity of β-MMC was at least 10 fold higher than that of α-MMC throughout the whole pH profile. Such activity on tRNA was also demonstrated by other RIPs like saporin, agrostin and luffin obtained from Sigma (data not shown).

In order to study the specificity of this reaction, polyhomoribonucleotides were used as the substrate. The results showed that the action of the MMCs was quite specific. By following the generation of acid soluble UV absorbing species, it was found that both MMCs preferred to act on polyU, but had only minimal activity on the other three polyhomoribonucleotides. To test whether the acid soluble products were small ribonucleolytic fragments or the free base, ion exchange chromatography was performed. All the UV absorbing species of the reacted mixture was adsorbed by the anion exchanger DEAE-Sepharose. Under the same experimental conditions, all the standard bases (A, C, G and U) would be in the DEAE breakthrough fraction while the oligoribonucleotides or the mononucleotides (AMP, CMP, GMP and UMP) would bound onto the DEAE-Sepharose. As no UV absorbance can be detected in the breakthrough fractions when the reaction product was passed through the column, it appears that all the products were ribonucleolytic fragments, but not the free bases.

Similar conclusion was obtained by following the time course of the reaction on the Mono-Q FPLC column. For the reaction of β-MMC on polyU, it was obvious that the polyribonucleotide was first cleaved into oligoribonucleotides of different size, as several different peaks were observed at intermediate reaction time upon chromatographic analysis. The oligoribonucleotides were then further cleaved to form the mononucleotide, UMP. No uracil could be observed and this suggested that the reaction involved ribonucleolytic, not N-glycosidic, cleavage. PolyU was cleaved at the phosphodiester

bond to give oligoribonucleotides and then finally into UMP. Such phosphodiester bond cleavage has also been reported with DNA as substrate. The cleavage is very specific. MMCs (Go *et al.*, 1992), TCS (Li *et al.*, 1992) and many other RIPs (Ling *et al.*, 1994) specifically cleave the supercoiled, double-stranded DNAs to produce relaxed-circular and linear forms of DNA.

While the potency of the N-glycosidase activity of both MMCs is similar, the ribonuclease activity of the two MMCs were significantly different. β-MMC was about 15 fold more active than α-MMC for the action on both tRNA and polyU. This might be related to the structural difference between the two MMCs. The two MMCs have similar but not identical, secondary and tertiary structures (Kubota *et al.*, 1986). Although the amino acid sequence of α-MMC have been determined (Ho *et al.*, 1991), that on β-MMC is not available. Nevertheless, N-terminal amino acid sequencing of β-MMC indicated that 10 out of 20 amino acids were different from those of α-MMC (Table 3.3) (Fong *et al.*, in press). The full sequence of β-MMC together with further characterization of the RNase activity are essential for explaining the observed difference in the catalytic efficiency of the two MMCs.

Table 3.3 Comparison of the N-terminal amino acid saquence of α- and β-MMCs (Fong *et al.*, in press).

	N-terminal amino acid sequence
α-MMC	**D V S F R L S G A D P R S Y G M F I K D**
β-MMC	**D V N F D L S T A T A K T Y T K F I E D**

Polyhomoribonucleotides has also been studied as substrate for another RIP, saporin (Barbieri *et al.*, 1994). It is found that adenine is released from polyA; however, no base formation can be detected with the other three polyhomoribonucleotides, polyC, polyG and polyU. Unfortunately, in that study, only the base formation is being monitored, and no attempt is made to investigate the ribonucleolytic cleavage. In the present investigation, by measuring the amount of acid-soluble UV absorbing species, it was clear that saporin reacted with both polyA and polyU. On the other hand, while α- and β-MMCs had a high activity on polyU, they also had a weak but not negligible activity on polyA. Therefore, it is necessary to determine whether there was ribonucleolytic activity or N-glycosidic activity in the reaction toward polyA and polyU.

The reaction product of saporin and polyA was first analyzed by TLC on PEI-cellulose plate. The result indicated that only adenine was released from the reaction and no AMP can be detected. The DEAE-Sepharose analysis of the reaction product also suggested that saporin can release adenine from polyA. In this regard, it is of interest to note that α- and β-MMC did not show any significant N-glycosidase activity on polyA, the minute acid soluble UV absorbing species were possibly the ribonucleolytic fragment but not adenine. On the other hand, saporin also reacted with polyU and the analysis of the reaction product suggested that no uracil was released. Therefore the reaction between saporin and polyU involved ribonucleolytic cleavage, whereas the reaction between saporin and polyA involved N-glycosidic cleavage.

To summarize, the present results indicated that α-MMC, β-MMC and saporin all exhibited significant RNase activity. Moreover, the activity showed substrate specificity. The presence of such RNase activity might have a common occurrence in RIP. Further study in this direction might provide insights into the metabolic role of RIPs in plant. For example, the occurrence of this RNase activity in RIPs might be responsible for the removal of the excess mRNA and tRNA in plant cell. The RIPs might also play a defensive role in the plant by degrading the RNA of the infected pathogens or virues through their RNase activities.

Chapter 4 PURIFICATION OF RNase-MC

4.1 Introduction

RNases are enzymes that cleave the phosphodiester bond in RNA. Although a vast amount of information has been accumulated on microbial and mammalian RNases, the plant RNases remains relatively un-explored. As shown in Chapter 3, α- and β-MMCs, and possibly many other plant RIPs, exhibited RNase activity. However, the specific activity was rather low and thus it is still unknown whether the RNase activity plays any significant role in the plant. Indeed, besides the RIP, the plant also has other much more potent RNase.

For example, from the seeds of *Momordica charantia* where the RIPs α- and β-MMCs are isolated, another protein also posssess RNase activity, it is named as RNase-MC (Ide *et al.*, 1991). Indeed, RNase-MC has been crystallized and its structure is studied in detail (De *et al.*, 1992). The molecular weight of RNase-MC is 22kDa, its sequence has also been determined (Ide *et al.*, 1991). However, the purification procedure has not been reported. More importantly, its kinetic properties have not been examined. Since the α- and β-MMCs purified in the present study also possessed RNase activity, therefore, it is of paramount importance to demonstrate that the RNase activity of MMCs is not due to its contamination by the RNase-MC. To this end, RNase-MC was purified and kinetically characterized.

The purification scheme employed was essentially the same as that used for the purification of the MMCs. The crude extract was passed through the anion exchanger DEAE-cellulose. An additional step of cation exchange chromatography on S-Sepharose was used to separate RNase-MC from the majority of the MMCs. The final purification was achieved by the Mono-S FPLC column (Fig. 4.1).

Using such three ion-exchange chromatographies procedure, RNase-MC was purified. Similar assays as described for α- and β-MMCs were performed. Such investigation, for example, the substrate specificity study, on the RNase-MC is very important for its potential applications in the field of molecular biology research, like the many other RNases from various sources which have different and restricted specificity towards different ribonucleotides.

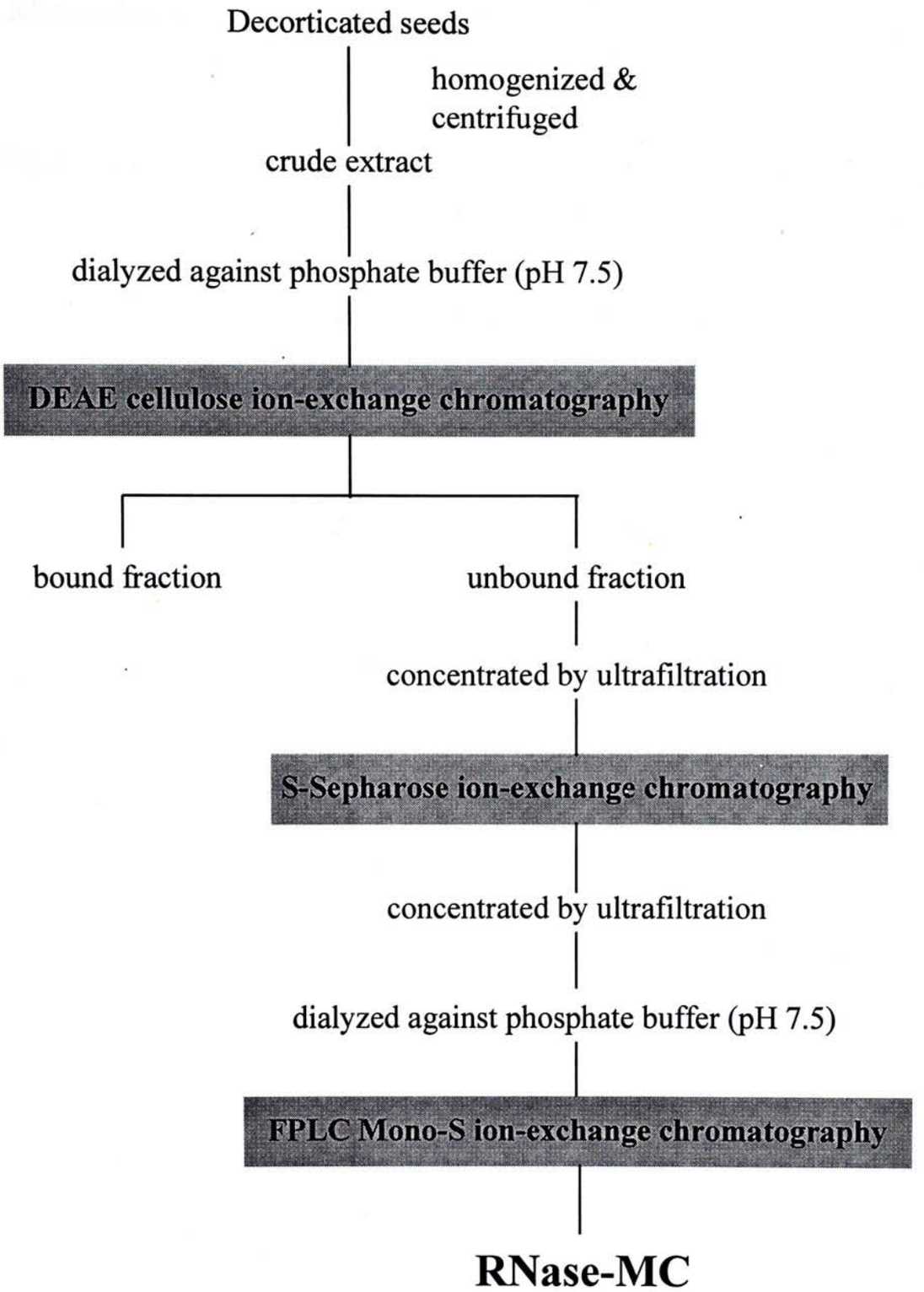

Fig. 4.1 The purification procedure for RNase-MC.

4.2 Material and Methods

4.2.1 Materials

S-Sepharose (Fast Flow) was purchased from Pharmacia Biotechnology. Silver Stain Plus kit and the Bradford Protein Assay kit were obtained from Bio-Rad. All the other materials were obtained from sources as described in Section 3.2.1. Similarly, for experiments involving RNA, all the buffers were pre-treated with 0.1% (v/v) DEPC for 18 hours at room temperature and then autoclaved to remove any traces of the chemical. All other reagents were of analytical or molecular biology grade and were used without further purification.

4.2.2 Purification of RNase-MC

Ripe dried seeds of *Momordica charantia* (bitter gourd) were purchased from the local market and stored in a cool dry place before use. Decorticated seeds were ground in a mortar. In a typical preparation, 10g of the seed powder was added to 100ml of 2mM sodium phosphate buffer, pH 7.5 and was homogenized by polytron (Kinematics). The slurry formed was stirred at 4°C overnight and then clarified by centrifugation (Beckman model J2-21) at 30,000*g* for 1 hour at 4°C. The pellet was discarded and the supernate was passed through two layers of cheesecloth to remove the large particles. Then the

filtered supernate was dialyzed against 2 litres of 2mM sodium phosphate, pH 7.5 overnight at 4°C with two changes of buffer.

The dialyzed sample was applied to a DEAE-cellulose column (Econo column, 5 x 15cm) pre-equilibrated with 2mM sodium phosphate, pH 7.5. After washing the column with the same buffer, the unbound fraction was collected and pooled together. The pooled fraction was then concentrated by ultrafiltration (Amicon, PM 10 membrane).

The concentrated sample was then applied to a S-Sepharose column (Econo-Column, 5 x 10cm) pre-equilibrated with 2mM sodium phosphate, pH 7.5. The column was washed with the same buffer, then eluted with a step gradient of 60mM NaCl in the same buffer. After the UV absorbance of the eluate decreases down to the baseline, the column was further eluted with another step gradient of 200mM sodium chloride in the same buffer.

The fractions with the highest RNase activity were pooled and named as S-Sepharose III. It was concentrated by ultrafiltration (Amicon, PM 10 membrane) and then dialyzed against 2 litres of 2mM sodium phosphate, pH 7.5 overnight at 4°C with two changes of buffer. The dialyzed sample was injected into the Mono-S FPLC column which has been equilibrated with 2mM sodium phosphate, pH 7.5. The proteins were first eluted with a linear gradient of 0-60mM sodium chloride in 30min., the sodium chloride

concentration was kept at 60mM for 10min., before another linear gradient of 60-200mM sodium chloride was applied in 60min. The peak with strong RNase activity was pooled accordingly and concentrated by ultrafiltration (Amicon, PM 10 membrane). It was then dialyzed against 2mM sodium phosphate, pH 7.5. The final concentrated proteins were aliquoted and stored at 4°C in sealed autoclaved microfuge tubes (Eppendorf).

4.2.3 RNase activity assay

The purification process was monitored by determining the RNase activity using tRNA as substrate, using the procedure as described in Section 3.2.6.

4.2.4 Protein Determination

Protein concentration was determined by the method of Bradford *et al.* (1976) using bovine serum albumin (BSA) as the standard. The microassay procedure, as described in the instruction of the Bradford Protein Assay kit of Bio-Rad, was used. Twenty μl of the sample or standard were mixed with 80μl of the reagent in a 96-well plate. The mixture was stand at room temperature for at least 10min. to allow the colour to develop. The absorbance at 595nm was measured using a microplate reader (Bio-Rad Model 3550).

4.2.5 Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis

Electrophoresis was performed according to the procedure of Laemmli and Favre (1973), using a 12% resolving gel and a 5% stacking gel, as described in Section 2.2.4. After electrophoresis, the gel was stained with the Silver Stain Plus kit (Bio-Rad).

4.2.6 Silver Staining

Silver staining was performed according to the instruction of the Silver Stain Plus kit from Bio-Rad. After SDS-PAGE, the gel was fixed in 200ml of fixing solution (1:1:3:5 v/v; acetic acid : fixative enhancer concentrate : water : methanol) for 30min. Then the gel was washed with 200ml of double distilled water for 10min. The washing step was repeated for another 10min. After washing, the gel was stained in the staining solution (50% development accelerator solution; 50% 1:1:1:7 v/v; silver complex solution : reduction moderator solution : image development reagent : water). The staining step was allowed to proceed until the desired intensity was obtained, which takes at least 5min. Afterwards, 5% acetic acid was added to terminate the staining step. The gel was immersed in acetic acid for 10min. and was then ready for drying and storage.

4.2.7 Activity of RNase-MC toward tRNA and Polyhomoribonucleotides

The experimental procedure was essentially the same as that described in Sections 3.2.6 and 3.2.7, excepted that the buffer used was 100mM Mes, pH 6.0 for the reaction of tRNA and polyhomoribonucleotides. One unit of enzyme activity is defined as the amount of enzyme that produce an absorbance increase at 260nm of one per min. in the acid soluble fraction per ml of reaction mixture under specified condition.

4.3 Results

4.3.1 Purification of RNase-MC

The first part of the purification procedure used was identical to that used for the purification of the MMCs, namely, the DEAE-cellulose chromatography. RNase-MC was eluted together with the MMCs in the breakthrough fraction of the anion exchanger. After concentration by ultrafiltration, the DEAE unbound fraction was applied onto the S-Sepharose column. The S-Sepharose ion-exchange chromatography was developed by a step-gradient method, using 60mM sodium chloride and then 200mM sodium chloride. The elution profile with 200mM sodium chloride indicated that besides the major peak (S-Sepharose I), two shoulders (S-Sepharose II, S-Sepharose III), although much smaller, can also be detected (Fig. 4.2). SDS-PAGE analysis showed that both S-Sepharose II and S-Sepharose III contained a band at 22kDa (Fig.4.3), representing RNase-MC. Comparing the two, S-Sepharose III had a higher specific RNase activity and was thus chosen for further purification.

The final purification of the RNase-MC was achieved by the Mono-S FPLC column, developed with two linear sodium chloride gradients. Three peaks were detected (Fig 4.4). Among them, the one eluted with a retention time of 73.6 min., corresponding to a sodium chloride concentration of 140mM, showed a very strong RNase activity.

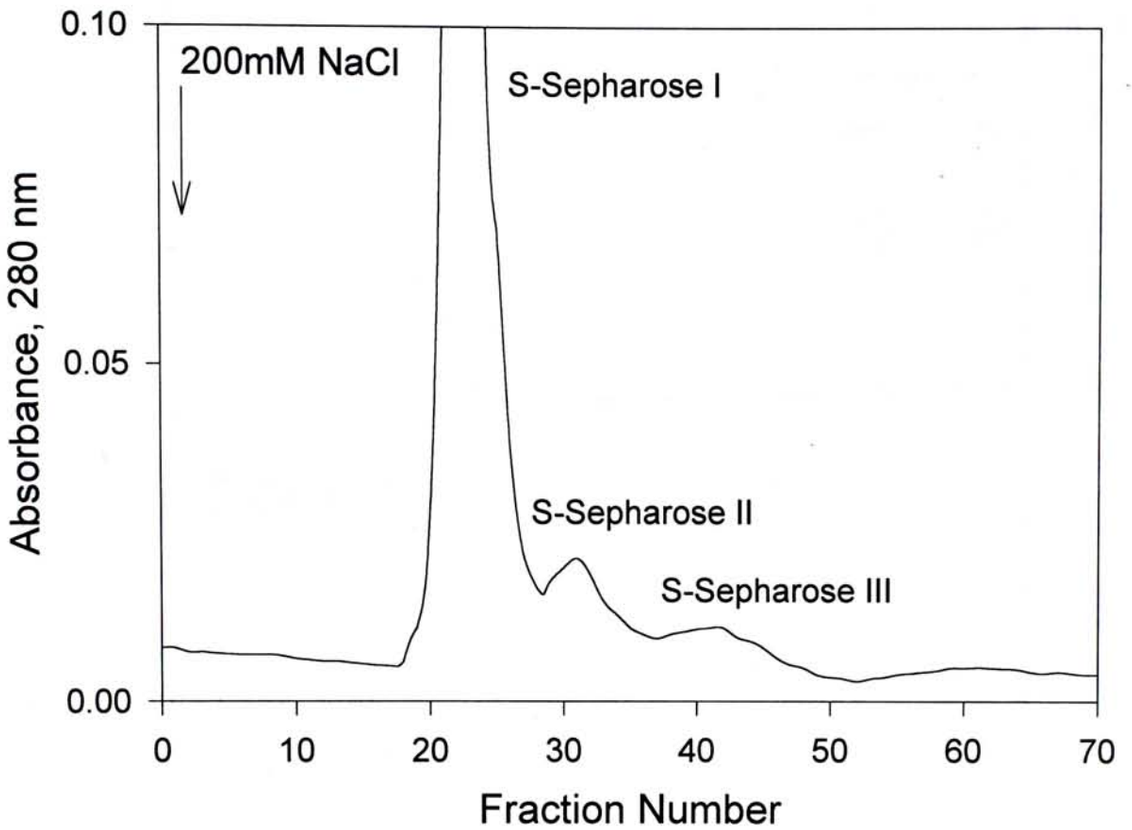

Fig. 4.2 Chromatography of the DEAE unbound fraction on S-Sepharose column. The unbound fraction of DEAE-cellulose column, after dialysis against 2mM sodium phosphate, pH 7.5, was loaded onto a S-Sepharose column (5 x 10cm) equilibrated with the dialyzing buffer. The column was first washed with the dialyzing buffer, followed with 60mM sodium chloride in 2mM sodium phosphate, pH 7.5. It was finally eluted with 200mM sodium chloride in the same buffer. This figure shows only the elution profile with 200mM sodium chloride. S-Sepharose I, S-Sepharose II and S-Sepharose III were collected for further analysis. Fraction of 5.5 ml were collected.

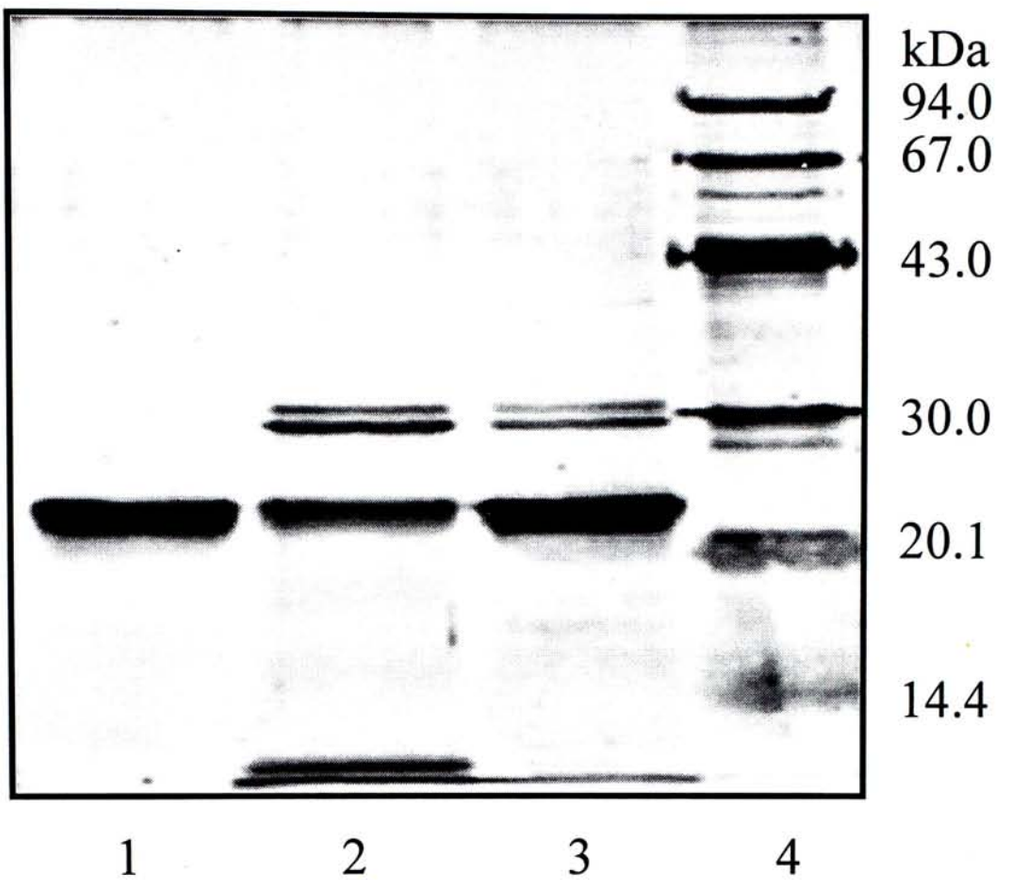

Fig. 4.3 SDS-PAGE of the sample at different stages of purification. Lanes 1: purified RNase-MC (Peak III from Mono-S column); lane 2: S-Sepharose II; lane 3: S-Sepharose III; lane 4: molecular weight standards (Pharmacia) including phosphorylase b (94.0kDa), bovine serum albumin (67.0kDa), ovalbumin (43.0kDa) , carbonic anhydrase (30.0kDa), soybean trypsin inhibitor (20.1kDa) and α-lactalbumin (14.4kDa).

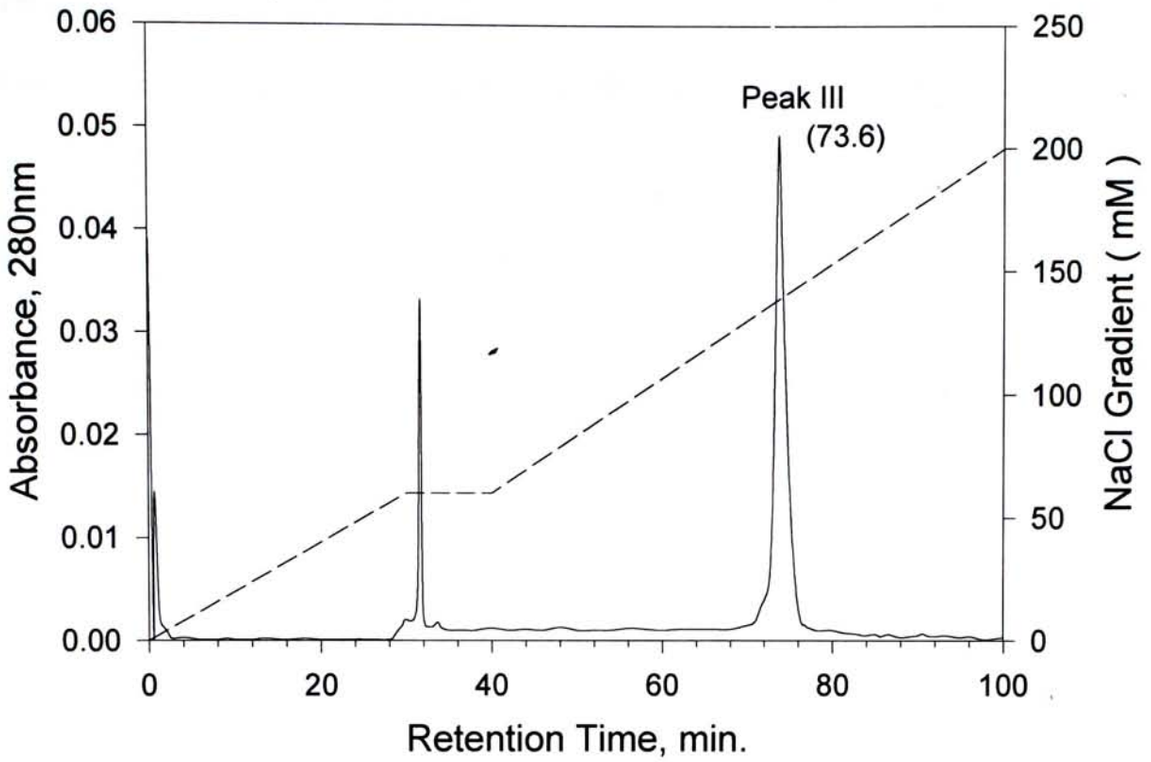

Fig. 4.4 Chromatography of S-Sepharose III on Mono-S FPLC column. S-Sepharose III, after concentration and dialysis, was applied to the column. The sample was first eluted with a 30min. linear gradient of 0-60mM sodium chloride in 2mM sodium phosphate, pH 7.5, the sodium chloride concentration was kept at 60mM for 10min. followed by another 60min. liear gradient of 60-200mM sodium chloride in the same buffer. Peak III was collected and identified as RNase-MC.

SDS-PAGE showed that the impurities originally present in S-Sepharose III had been successfully removed by the Mono-S column and this peak III showed only one single band of molecular weight about 22kDa. Thus, RNase-MC was purified.

4.3.2 Activity of RNase-MC toward tRNA and Polyhomoribonucleotides

RNase-MC was found to act catalytically on tRNA, as shown by the release of acid-soluble UV absorbing species. The activity varied with the pH of the reaction mixture. Maximal activity was observed at pH around 6 (Fig. 4.5). Substrate specificity study on the polyhomoribonucleotides indicated that RNase-MC acted on both polyC and polyU, but it showed very low activity towards polyA and polyG (Table 3.1). Comparing polyC and polyU, it was found that the activity of RNase-MC toward polyC was about 8 fold lower than that toward polyU. To confirm that the acid-soluble UV absorbing species were oligo- or monoribonucleotides, the product from the reaction between RNase-MC and polyU was analyzed by DEAE-Sepharose. All the UV absorbing species was adsorbed (Table. 3.2) onto the column and hence confirmed that no free uracil was released in the process.

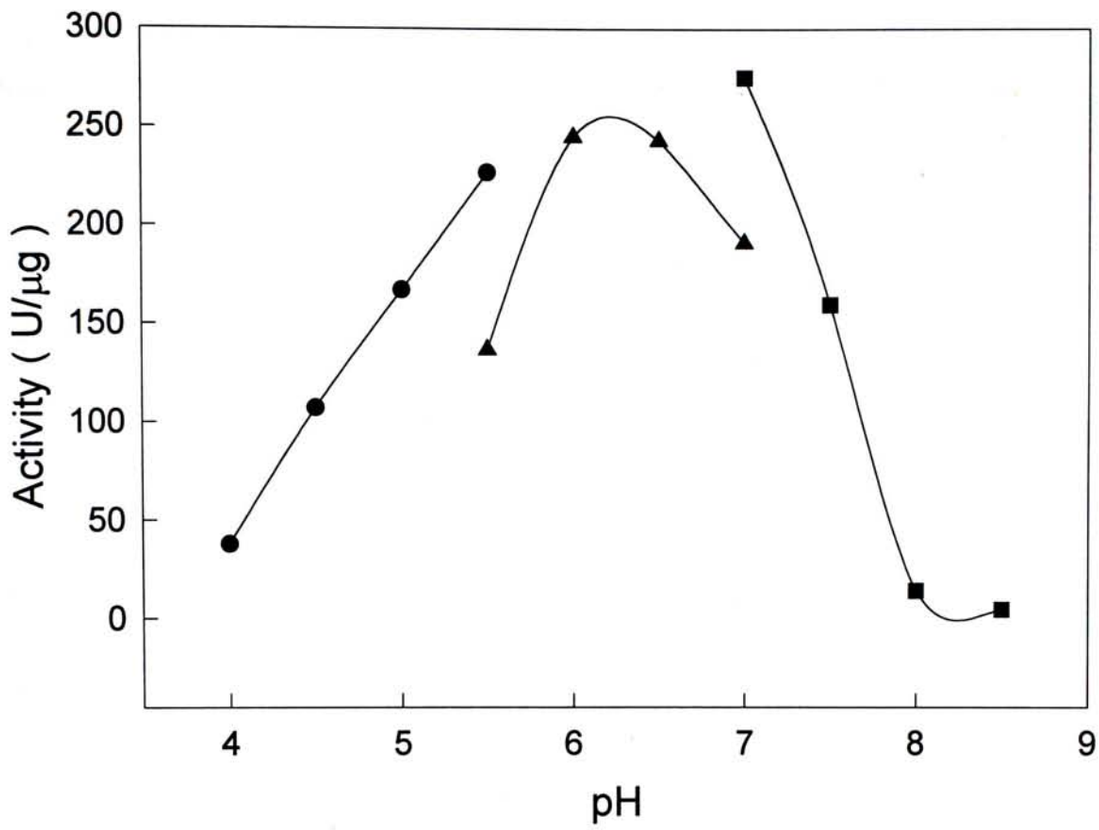

Fig. 4.5 Effect of pH on RNase-MC's action on tRNA. RNase-MC was incubated with 200μg of tRNA in a final volume of 150μl 0.1M sodium acetate (●), Mes (▲) or Hepes (■) at 37°C for 30min. The reaction was stopped by the addition of 350μl of ice-cold 3.4% perchloric acid. After standing on ice for 15min., the sample was centrifuged at 15,000*g* for 15min. at 4°C. The absorbance of the supernatant, after suitable dilution, was measured at 260nm.

Table 4.1 Activities of RNase-MC on tRNA and various polyhomoribonucleotides[a].

Substrate	Activity (U/μg protein)
tRNA	244.77
poly A	8.54
poly C	34.15
poly G	2.85
poly U	271.73

[a]The activity was determined as described in Section.4.2.7.

Table 4.2 Comparison of the amount of UV absorbing species after reaction using the precipitation assay and the DEAE analysis.

Reaction mixture	Total Absorbance[b] 260nm	
	acid soluble	DEAE breakthrough
(A) Reaction[a]		
RNase-MC + polyU	1.428	0.05
(B) Standards		
U	+[c]	+
UMP	+	-
polyU	-	-

[a]The assays were performed as described in Section. 3.2.7. The amount of RNase MC used was 0.0078 μg.

[b]The total absorbance is obtained by multiplying the absorbance with the total volume (ml).

[c] "+" indicates the presence of >95% and "-" indicates the presence of <5% of the sample added.

4.4 Discussion

The present purification scheme is useful for the purification of RNase-MC from the seeds of *Momordica charantia*. This scheme consisted of three successive ion-exchange chromatography steps, the first one was the anion exchanger DEAE-cellulose, followed by the two cation exchange steps, namely, S-Sepharose and Mono-S FPLC column. Although pure RNase-MC can be obtained; however, compared with that for the MMCs, the present purification procedure suffers from the disadvantage that the time required was much longer as the number of chromatographic steps and dialysis was increased. The whole purification process took about one week to finish. Such prolonged process may result in the loss of activity of the protein, the extra dialyzing steps also result in considerable precipitation of the protein. Hence, it is desirable to further reduce the number of chromatographic steps as well as the dialyzing steps in order to have a better recovery of the protein.

From the present purification scheme, only about 30μg of RNase-MC could be purified from 10g of seed powder, the yield was by no means satisfactory. At the moment, it is not known whether the low amount of RNase-MC obtained was due to the loss during the purification process, or that the original amount of the RNase-MC present in the seeds is in such a minute quantity. If the latter case is the real situation, then,

obviously, it is necessary to increase the amount of starting material in order to obtain more purified RNase-MC from the seed.

The loss in the purification process should be minimized. To reduce the loss of the protein, the number of chromatographic steps should be reduced. Among the three chromatography steps, both S-Sepharose and Mono-S involve cation exchange, indeed, the functional group of the exchanger is the same. Therefore, theoretically, only one is sufficient, and this can also decrease the number of dialysis. Unfortunately, RNase-MC represents only a minute percentage of the basic protein in the seed extract of *Momordica charantia.* The majority are the MMCs which co-exist with RNase-MC in the DEAE breakthrough fraction. Consequently, although the high resolving power of the Mono-S FPLC column is capable of removing the MMCs from RNase-MC, however, the amount of protein that should be loaded onto the column in order to obtain a significant amount of RNase-MC exceeds the capacity of the FPLC column. On the other hand, although a much larger S-Sepharose column can be used, however, the resolution of the gel is not good enough to obtain RNase-MC in one step. Consequently, two successive cation exchange steps, the high capacity S-Sepharose and the high resolution Mono-S FPLC, seems to be essential for the purification of RNase-MC.

The purified RNase-MC acted catalytically on tRNA and polyhomoribonucleotides. The activity involved the cleavage of the phosphodiester bond

from the ribonucleotides as shown from the result of product analysis on DEAE-Sepharose. This activity was very strong and worked at nanomolar concentration of the enzyme. The activity was also very specific and acted preferentially on polyU. It also acted on polyC but the activity was at least 8 fold lower than that toward polyU. This was quite similar to the unpublished finding of Ide *et al.* (1991) that RNase-MC has a remarkably high specificity toward the dinucleoside monophosphates CpU, ApU and UpU.

Comparsion of the amino acid sequence of RNase-MC with those of RNase T2 (Kawata *et al.*, 1988) and RNase-RH (Horiuchi *et al.*, 1988), base non-specific RNases from fungi, shows only a low degree of homology (approx. 28%). When compared to the sequence of the plant RNases from *Nicotiana alata*, the S-glycoproteins S2, S3 and S6 (McClure *et al.*, 1989), the sequence homology is much higher (approx. 41%). There are two highly conserved segments between RNase-MC and these S-glycoproteins, also, there are other individual conserved positions among these RNases, all eight cysteine residues in RNase-MC are totally conserved with that of the S-glycoproteins. Therefore, the amino acid sequence of RNase-MC is quite similar with that of the plant RNase S-glycoproteins. The molecular weight of RNase-MC is also similar to that of the RNase-LE (Jost *et al.*, 1991), a RNase isolated from the cultured tomato (*Lycopersicon esculentum*) cells, which is around 22kDa, however, the isoelectric points (pI) of these two plant RNase are very different from each other, the pI of RNase-MC is about 9.8 but

that of the RNase-LE is about 4.2. Despite the difference in the pI between these two RNases, the pH optimum of their RNase activities are very similar, pH 5.5 for RNase-LE and pH 6.0 for RNase-MC. On the other hand, the pH optimum for S-glycoproteins is around pH 7.0. Such differences may be accounted for by the unconserved amino acid sequence of these RNases, however, further study is obviously needed to study the enzymatic activity, physical and chemical properties of the plant RNases.

When RNase-MC is compared with α- and β-MMCs, their physical properties were different from each other. The molecular weight of RNase-MC was about 22kDa but that of both MMCs were about 30kDa. Their pI were also different, the pI of α- and β-MMCs were about 8.5-9.0 while that of the RNase-MC was much higher, about 9.8. This could be observed from the elution profile on the Mono-S FPLC chromatography, RNase-MC was eluted with a much higher sodium chloride concentration than that of both MMCs. Moreover, the pH rate profiles of RNase-MC and the MMCs was also slightly different, the pH optimum of both MMCs was pH 5.5, while that of RNase-MC was pH 6.0. RNaes-MC acted on both poly C and poly U with the activity towards poly U about eight fold higher than that towards poly C, however, β-MMCs acted only on poly U, suggesting that the substrate specificity of RNase-MC was different from the MMCs. From these information, it was suggested that although MMCs and RNase-MC possessed similar RNase activity, they were not identical as their substrate specificity was different.

Although the RNase activity of MMCs and RNase-MC was not identical, nevertheless, they were very similar to each other. The only major difference is that RNase-MC was at least a thousand fold more active than the MMCs. Consequently, it is necessary to confirm whether the RNase activity observed for the MMCs was an intrinsic property of the proteins, or whether it was due to a minor contamination of the very active RNase-MC. Two evidences supported the first hypothesis. Firstly, SDS-PAGE analysis of the MMC preparation, even with prolonged staining with the Silver Staining reagent, failed to detect any impurities (data not shown). Secondly, RNase activity analysis of the fraction obtained from Mono-S column during the purification of the MMCs correlated well with the protein profile of α- and β-MMC (Fig. 4.6). Besides these two peaks of RNase activity, there was an additional and much stronger peak of activity, representing RNase-MC. This strong activity peak was completely separated from those of the MMCs; indeed, with baseline separation. Consequently, it was believed that the MMCs did possess an intrinsic RNase activity even though it was week.

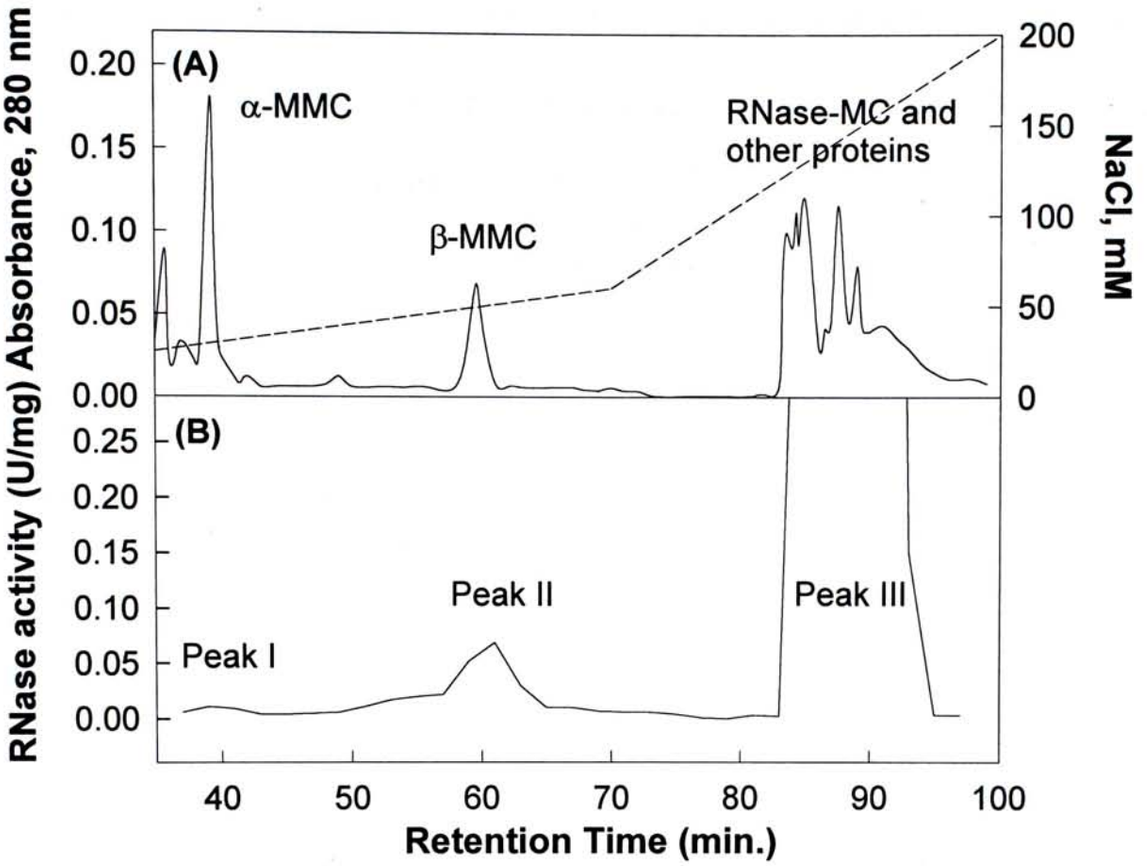

Fig. 4.6 Chromatography of DEAE-I on Mono-S FPLC column. The eluate was monitored for both UV absorbance at 280nm (A) and RNase activity with tRNA as substrate (B).

To sum up, a purification scheme was developed for RNase-MC; however, further improvment is needed to increase the yield of the purification process and to reduce the purification time. Since RNase-MC possessed a very high and specific activity, it might be very useful in molecular biology research.

Chapter 5 Conclusion

Conclusion

Ribosome-inactivating proteins from the bitter gourd (*Momordica charantia*) was first isolated by Yeung *et al.*, (1985) and named as α- and β-MMCs. The purification scheme at that time required quite a lot of time to complete and the yield of the proteins is also very low. Two modifications of the original procedure were proposed subsequently (Go *et al.*, 1992 and Poon 1994). Both the purification time and the yield of the protein has been improved. In the present study, the procedure of Poon (1994) was adopted. Two ion-exchange chromatographies were used: DEAE-cellulose and Mono-S FPLC chromatography. The application of these two chromatographic steps has simplified the purification procedure and the purification time was reduced from at least a week to two days, while the yield of the protein remained satisfactory.

Both the purified α- and β-MMCs possess N-glycosidase activity (Poon, 1994). In the present study, it was demonstrated that this N-glycosidase activity depended on the presence of the ribosomal proteins for their action. In the course of this study, it was also found out that both MMCs possessed RNase activity and cleaved the naked ribosomal RNA into a smear. Further investigation of this RNase activity of MMCs was carried out by using tRNA and polyhomoribonucleotides (polyA, polyC, polyG and polyU). The result showed that both MMCs acted on tRNA and among the polyhomoribonucleotides, it acted only on polyU. The activity of β-MMCs was at least about 15 fold higher than

that of α-MMC. The same assay was carried out for other RIPs, such as saporin, luffin and agarostin. All these RIPs showed the RNase activity. This suggested that RNase activity may have a wide occurrence in RIPs.

Together with the well-known N-glycosidase activity (Endo *et al.*, 1988), the DNase activity (Go *et al.*, 1992; Ling *et al.*, 1994) and the RNase activity discovered in the present study, there were at least three different enzymatic properties of the RIPs. As the RIPs have been shown to possess many different biological activities, such as abortifacient activity, anti-tumor and immunosuppressive activity, therefore, it is important to study the correlation between these three enzymatic properties with the various biological properties of the RIPs.

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